AA

PCT

WO   9605303A1

INTERNATIONAL APPLICATION PUBLISHED UNDER T.

(51) International Patent Classification [6] : C12N 15/12, C07K 14/705, A61K 39/395, C12N 15/11, A61K 38/17, C07K 16/28, G01N 33/68	**A1**	(11) International Publication Number: **WO 96/05303** (43) International Publication Date: 22 February 1996 (22.02.96)

(21) International Application Number: PCT/US95/09636

(22) International Filing Date: 9 August 1995 (09.08.95)

(30) Priority Data:
    289,699   12 August 1994 (12.08.94)   US

(71) Applicant: OKLAHOMA MEDICAL RESEARCH FOUNDA-TION [US/US]; 825 N.E. Thirteenth Street, Oklahoma City, OK 73104 (US).

(72) Inventors: FUKUDOME, Kenji; 13125 West Park Place, Oklahoma City, OK 73142 (US). ESMON, Charles, T.; 5800 North Stonewall, Oklahoma City, OK 73111 (US).

(74) Agent: PABST, Patrea, L.; Arnall Golden & Gregory, 2800 One Atlantic Center, 1201 West Peachtree Street, Atlanta, GA 30309-3450 (US).

(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: CLONING AND REGULATION OF AN ENDOTHELIAL CELL PROTEIN C/ACTIVATED PROTEIN C RECEPTOR

(57) Abstract

Human protein C and activated protein C were shown to bind to endothelium specifically, selectively and saturably (Kd=30 nM, 7000 sites per cell) in a Ca^{2+} dependent fashion. Expression cloning revealed a 1.3 kb cDNA that coded for a novel type I transmembrane glycoprotein capable of binding protein C. This protein appears to be a member of the CD1/MHC superfamily. Like thrombomodulin, the receptor involved in protein C activation, the endothelial cell protein C receptor (EPCR) function and message are both down regulated by exposure of endothelium to TNF. Identification of EPCR as a member of the CD1/MHC superfamily provides insights into the role f protein C in regulating the inflammatory response, and determination of methods for pharmaceutical use in manipulating the inflammatory response.

CLONING AND REGULATION OF AN ENDOTHELIAL CELL PROTEIN C/ACTIVATED PROTEIN C RECEPTOR

Background of the Invention

The present invention is generally in the area of cloning, expression, and regulation of an endothelial cell protein C/activated protein C

5   receptor.

Protein C plays a major role in the regulation of blood coagulation. Patients deficient in protein C usually exhibit life threatening thrombotic-complications in infancy

10   (Seligsohn et al., (1984) N. Engl. J. Med. 310, 559-562; Esmon, (1992) Trends Cardiovasc. Med. 2, 214-220) that are corrected by protein C administration (Dreyfus et al., (1991) N. Engl. J. Med. 325, 1565-1568). In addition, activated

15   protein C (APC) can prevent the lethal effects of E. coli in baboon models of gram negative sepsis (Taylor et al., (1987) J. Clin. Invest. 79; U.S. Patent No. 5,009,889 to Taylor and Esmon) and preliminary clinical results suggest that protein C

20   is effective in treating certain forms of human septic shock (Gerson et al., (1993) Pediatrics 91, 418-422). These results suggest that protein C may both control coagulation and influence inflammation. Indeed, inhibition of protein S, an

25   important component of the protein C pathway, exacerbates the response of primates to sublethal levels of E. coli and augments the appearance of TNF in the circulation (Taylor et al., (1991) Blood 78, 357-363). The mechanisms involved in

30   controlling the inflammatory response remain unknown.

Protein C is activated when thrombin, the terminal enzyme of the coagulation system, binds to an endothelial cell surface protein, thrombomodulin

35   (Esmon, (1989) J. Biol. Chem. 264, 4743-4746; Dittman and Majerus, (1990) Blood 75, 329-336;

Dittman, (1991) _Trends Cardiovasc. Med._ 1,
331-336). In cell culture, thrombomodulin
transcription is blocked by exposure of endothelial
cells to tumor necrosis factor (TNF) (Conway and
5 Rosenberg, (1988) _Mol. Cell. Biol._ 8, 5588-5592)
and thrombomodulin activity and antigen are
subsequently internalized and degraded (Lentz et
al., (1991) _Blood_ 77, 543-550, Moore,K.L., et.al.,
(1989) _Blood_ 73, 159-165). In addition, C4bBP,
10 a regulatory protein of the complement system,
binds protein S to form a complex that is
functionally inactive in supporting APC
anticoagulant activity _in vitro_ (Dahlbäck, (1986)
J. Biol. Chem. 261, 12022-12027) and _in vivo_
15 (Taylor, et al., 1991). Furthermore, C4bBP behaves
as an acute phase reactant (Dahlbäck, (1991)
Thromb. Haemostas. 66, 49-61). Thus, proteins of
this pathway not only appear to regulate
inflammation, but they also interact with
20 components that regulate inflammation, and they
themselves are subject to down regulation by
inflammatory mediators.

Given the central role of the protein C
pathway in regulating the host response to
25 inflammation and the critical role of the pathway
in controlling blood coagulation, it is important
to identify and characterize all of the components
that interact with the system. This is especially
true since the molecular basis of the
30 anti-inflammatory effects of the protein C pathway
components have yet to be elucidated at the
molecular level.

It is therefore an object of the present
invention to provide a cellular receptor for
35 protein C and activated protein C.

It is a further object of the present
invention to provide nucleotide sequences encoding

3

the cellular receptor and amino acid
characterization of the receptor which allows
expression of recombinant native and modified forms
of the receptor.

5   It is another object of the present
invention to provide methods of modulating the
inflammatory response involving protein C and
activated protein C.

Summary of the Invention

10   An endothelial cell protein C binding
protein (referred to herein as "EPCR") has been
cloned and characterized. The protein is predicted
to consist of 238 amino acids, which includes a 15
amino acid signal sequence at the N-terminus, and a

15   23 amino acid transmembrane region which
characterizes the receptor as a type 1
transmembrane protein. The protein binds with high
affinity to both protein C and activated protein C
(Kd=30 nM) and is calcium dependent. The message

20   and binding function of the receptor are both down
regulated by cytokines such as TNF.

These results identify a new member of a
complex pathway that, like other members of the
pathway, is subject to regulation by inflammatory

25   cytokines, and can therefore be used to modulate
inflammatory reactions in which protein C or
activated protein C is involved. Inhibition of
the inflammatory response can be obtained by
infusing soluble EPCR. Alternatively, localizing

30   EPCR to surfaces in contact with blood will render
the surfaces anticoagulant by virtue of the ability
of EPCR to bind and concentrate the anticoagulant
activated protein C at the surface. Alternatively,
the function of EPCR can be enhanced by

35   overexpressing the EPCR in endothelium that could

4

be used to coat vascular grafts in patients with
vascular disease or on stents in cardiac patients.

Brief Description of the Drawings

5   Figures 1A, 1B and 1C are flow cytometric
analyses of Fl-APC (fluorescent labelled activated
protein C) binding to HUVEC (human umbilical vein
endothelial cells). Figure 1A is a graph of cell
number versus log of fluorescence intensity,
demonstrating Fl-APC binding to HUVEC. HUVEC (1 x
10   10^5) were incubated at room temperature without
(dotted line) or with 160 nM of Fl-APC (solid line)
in the presence of 1.3 mM $CaCl_2$. After washing,
bound APC was analyzed by flow cytometry. Figure
1B is a graph of fluorescence intensity versus Fl-
15   APC concentration (nM) demonstrating the
concentration dependence of Fl-APC binding to
HUVEC. HUVEC were incubated with Fl-APC in the
absence (open circles) or presence of 1.3 mM $CaCl_2$
(closed circles) and binding was measured as in A.
20   Mean channel fluorescence intensity is plotted for
each Fl-APC concentration (between 0 and 800 nM).
Figure 1C is a graph of the percent of mean
fluorescence versus unlabeled protein concentration
($\mu g/ml$), demonstrating the effects of unlabeled
25   proteins on Fl-APC binding to HUVEC. Fl-APC
binding to HUVEC was carried out in the presence of
the indicated concentrations (between 0 and 100
$\mu g/ml$) of unlabeled APC, protein C, protein S,
factor X and Xa or recombinant Gla-domainless
30   protein C (rGDPC).

Figures 2A, 2B, 2C and 2D are graphs of
^{125}I-APC Binding to HUVEC Monolayers. Figure 2A is
a graph of the bound APC (cpm x 10^{-3}) versus time
(min), showing the time course of ^{125}I-APC binding
35   to HUVEC. HUVEC monolayers (1.2 x 10^5 cells) were
incubated at 4°C with 32 nM (filled squares) or 8

nM (open squares) ^{125}I-APC. At the indicated times,
cells were washed and bound radioactivity was
measured. Figure 2B is a graph of bound APC (cpm x
10^{-3}) versus unlabeled protein (nM) demonstrating

5   the effects of unlabeled APC and rGDPC on ^{125}I-APC
binding to HUVEC. HUVEC were incubated at 4°C for
one hour with ^{125}I-labeled APC in the presence of
the indicated concentrations (between 01 and
approximately 1000 nM) of unlabeled APC (open

10 circles) or rGDPC (closed circles). After washing,
bound radioactivity was measured. Figure 2C is a
graph of bound APC (fmol/well) versus free APC (nM)
demonstrating the concentration dependence of [125]I-
APC binding to HUVEC. Monolayers of HUVEC were

15 incubated with the concentrations of ^{125}I-APC
indicated as described above. Specific binding was
determined as described below. Figure 2D is a
Scatchard analysis of ^{125}I-APC binding to HUVEC.
Each value was calculated from the data shown in

20 Figure 2C.
            Figures 3A and 3B are flow cytometric
analyses of Fl-APC binding to 293T cells
transfected with a cDNA clone of EPCR. Cells were
transfected with a clone EPCR/pEF-BOS or pEF-BOS

25 (negative control) by the calcium/phosphate method.
After 24 h, cells were harvested and Fl-APC binding
was performed in the absence (dotted lines) or
presence of 1.3 mM $CaCl_2$ (solid lines).
            Figures 4a-4c are the predicted protein

30 structure of EPCR based on nucleotide sequence (SEQ
ID NO. 1), predicted amino acid sequence (SEQ ID
NO. 2) and a hydropathy plot of EPCR. The signal
sequence and transmembrane region are indicated
with the solid bars.

35   Figures 5a-5b are a comparison of the amino
acid sequence of EPCR to the amino acid sequences
of other members of the CD1 family and CCD41. The
EPCR sequence is shown in the first line and

SUBSTITUTE SHEET (RULE 26)

6

compared to murine CCD41 (second line), human CD1d
(third line) and murine CD1.2 (fourth line).
Identities with EPCR are indicated by open boxes.
Residues that are conserved between EPCR and all of
5   the human CD1 family members are indicated by a
double asterisk. Residues shared with one or more
members of the CD1 family are indicated by a single
asterisk.

        Figure 6 is a comparison of the amino
10   acid sequence of human EPCR (first line) to the
amino acid sequence of murine EPCR (second line).
Identities are indicated by lines. Similarities
are indicated with dots.

        Detailed Description of the Invention
15   I.   **Cloning and Characterization of EPCR.**
        Human protein C and activated protein C
are shown to bind to endothelium specifically,
selectively and saturably (Kd = 30 nM, 7000 sites
per cell) in a Ca^{2+} dependent fashion. FL-APC
20   binding to various human cell lines were examined,
and found that the binding was HUVEC specific. A
human kidney cell line transformed with SV40 large
T antigen, 293T cells, expressed very few of these
binding sites. A HUVEC cDNA library was
25   constructed using the powerful mammalian expression
vector, pEF-BOS (Mizushima and Nagata, (1990)
Nucleic Acids Res. 18, 5322). Plasmid DNA was
prepared from subpools of independent colonies
(2,500 colonies per pool), and transfected into
30   293T cells, using the method of Kaisho et al.,
(1994) Proc. Natl. Acad. Sci. (USA) 91, 5325.
FL-APC binding was analyzed on a flow cytometer.
One of eight subpools gave a positive signal. This
subpool was divided into 20 subpools and
35   rescreened. After three rounds of enrichment, one
positive clone, EPCR-1, was isolated. EPCR-1

SUBSTITUTE SHEET (RULE 26)

carries a 1.3 kb insert. When transfected into
293T cells, this clone was capable of expressing
the calcium-dependent binding site for FL-APC on
the 293T cell surface.

5   Expression cloning revealed a 1.3 kb cDNA
that coded for a type I transmembrane glycoprotein
capable of binding protein C. This protein appears
to be a member of the CD1/MHC superfamily. Like
thrombomodulin, the receptor involved in protein C
10   activation, the endothelial cell protein C receptor
(EPCR) function and message are both down regulated
by exposure of endothelium to TNF. Identification
of EPCR as a member of the CD1/MHC superfamily
provides insights into the role of this receptor
15   for protein C in regulating the inflammatory
response.

Materials and Methods

Protein Preparation

Human protein C (Esmon et al., (1993)
20   Meths. Enzymol. 222, 359-385), APC (Esmon et al.,
1993), recombinant gla domainless protein C (rGDPC)
(Rezaie et al., (1992) J. Biol. Chem. 267,
11701-11704), protein S (Taylor et al., 1991),
factor X and factor Xa (Le Bonniec et al., (1992)
25   J. Biol. Chem. 267, 6970-6976) were prepared as
described in the cited publications.

Selective labeling of the active site of
APC with fluorescein was performed by the method of
Bock (Bock, P.E. (1988) Biochemistry 27,
30   6633-6639). In brief, N$^{\alpha}$—
[(acetylthio)acetyl]-D-Phe-Pro-Arg-CH$_2$Cl (200 μM)
was reacted with 40 μM APC for 1 hour at room
temperature. After dialysis, the covalently
modified APC was incubated at room temperature for
35   one hour with 200 μM 5-(iodoacetamido)fluorescein
(Molecular Probes). Free fluorescein was removed
by gel filtration on a PD-10 column (Pharmacia).

8

With this method, each molecule of fluoresceinated
APC (Fl-APC) contains a single dye at the active
site and hence all of the fluorescent molecules
behave identically.

5   Iodogen (Pierce) was used to radiolabel
APC with Na[^{125}I] (Amersham) according to the
manufacture's protocol in the presence of 5 mM
CaCl$_2$. Free ^{125}I was removed by gel filtration on a
PD-10 column. The specific activity of the ^{125}I-APC
10   was 1 x 10^4 cpm/ng protein.

Cell Culture

Human umbilical vein endothelial cells
(HUVEC) were isolated from fresh umbilical-cords by
collagenase treatment and cultured in medium 199
15   containing 15% fetal bovine serum, 10 µg/ml
heparin, and 0.5% endothelial cell growth
supplement prepared from bovine brain extract
(Maciag at al., (1979) _Proc. Natl. Acad. Sci. (USA)_
_76_, 5674-5678). HOS (ATCC CRL 1543), HEp-2 (ATCC
20   CCL 23) and 293 cells (ATCC CRL 1573) transformed
with SV40 large T antigen (293T, a gift from Dr.
Kenji Oritani) were maintained in Earl's MEM
supplemented with 10% fetal bovine serum. The
human lymphocyte cell lines, Jurkat, MOLT3 (ATCC
25   CRL 1552), Jijoye (ATCC CCL 87), Raji (ATCC CCL
86), U-937 (ATCC CRL 1593), HL-60 (ATCC CCL 240),
and HEL (ATCC TIB 180), were maintained in
RPMI-1640 medium supplemented with 10% fetal bovine
serum.

30   **Flow Cytometric Analysis of Fl-APC**
Binding to Cells

Adherent cells were harvested by
incubation at 37°C for 5 min in phosphate buffered
saline (PBS) containing 0.02% EDTA. Cells were
35   washed twice with EDTA/PBS and then once with
Hank's balanced salt solution (HBSS). They were
resuspended in HBSS containing 1% bovine serum
albumin (BSA) and 0.02% sodium azide (binding

9

buffer). Cells (1 x 10⁵) were incubated at room
temperature for 45 min with Fl-APC in the dark.
After washing, they were resuspended in the binding
buffer containing 0.5 µg/ml of propidium iodide.

5   Bound Fl-APC was analyzed on a flow cytometer,
FACScan (Becton Dickinson). Living cells were
gated on a dot plot display of forward-scatter
(FSC) versus fluorescence-2 (FL2), and Fl-APC
binding was detected on the fluorescence-1 (FL-1)

10 channel. All experiments were performed in
duplicate.

¹²⁵I-APC binding to HUVEC

Monolayers of HUVEC in 24-well
microplates (Costar) (1 x 10⁵ cells per well) were

15 washed twice with EDTA/PBS and once with ice-cold
HBSS. Cells were then incubated at 4°C for one
hour in the binding buffer with ¹²⁵I-APC. After
washing three times with ice-cold HBSS, cells were
released with the EDTA buffer, and the bound

20 radioactivity was measured in a gamma counter
(Isodata 500). To determine non-specific,
calcium-independent adsorption of radioactivity,
the cells were washed with EDTA/PBS and residual
radioactivity in the cell pellet was measured.

25 Non-specific binding of radioactivity was
consistently less than 5% of the specific binding.
The data was analyzed using the Enzfitter program
(Elsevier Biosoft, Cambridge, U.K.).

Construction of HUVEC cDNA Library

30 Poly-A RNA was isolated from HUVEC (1 x
10⁸ cells) using the FastTrack™ mRNA isolation kit
(Invitrogen). cDNA was synthesized from 3 µg of
poly-A RNA using a Librarian™ I kit (Invitrogen).
A BstX I adaptor was ligated, double stranded cDNA

35 was fractionated by agarose gel electrophoresis,
and cDNA longer than 700 bp was ligated into a
mammalian expression vector, pEF-BOS (Mizushima and

10

Nagata, 1990; this vector was a kind gift from Dr.
S. Nagata). The construct was transfected into *E.
coli* DH1OB by electroporation (Bio-Rad Gene
Pulser™). The library consisted of 8 X 10⁶
5   independent colonies with an average size of 2.0
kb.

Expression Cloning and Sequence Analysis

Approximately 2×10^4 independent colonies
were divided into eight subpools (each containing
10   2,500 independent colonies) and plasmid DNA was
prepared from each subpool. Sub-confluent 293T
cells in 24-well microplates were transfected with
1 μg of the DNA by the calcium/phosphate method
(Graham and Van Der Eb, (1973) <u>Virology</u> 52, 456-
15   467). After 20 hours, the medium was changed, and
culture was continued for another 24 hours. The
subpools were screened for F1-APC binding by FACS
analysis as described above. The positive library
pool was then divided into 20 new pools and
20   rescreened. After three rounds of screening, 96
individual clones were tested and one positive
clone was identified.

The insert (1.3 kb) was subcloned into
pBluescript™ (Stratagene), and the nucleotide
25   sequence was determined using a Sequenase™ version
2.0 DNA Sequencing kit (USB). Nucleotide and
protein database search employed the BLAST™ (NCBI)
and FASTA™ programs (GCG) with GenBank, EMBL, and
SwissProt databases.

30   **Northern Blot Analysis**

Total RNAs (15 μg) from various cells
were isolated, electrophoresed through formaldehyde
agarose gels and transferred to a nylon membrane
(Hybond-N™, Amersham). The 483 bp *Xba* I fragment
35   from the 5' end of the EPCR cDNA was labeled by
random priming according to the manufacturer's

instructions (Multiprime™ DNA labeling system,
Amersham) and used for hybridization.

        Protein C and APC Binding to HUVEC

        Endothelial cells in suspension bound
5   FL-APC, as monitored by flow cytometry, and
demonstrated in Figure 1A. Binding was saturable
and Ca^{2+} dependent, as shown by Figure 1B. Optimal
binding required at least 1 mM Ca^{2+}. FL-APC was
displaced from the cell surface by APC and protein
10 C equivalently, as shown by Figure 1C. The
homologous Gla-domain containing proteins, protein
S, factor X, and its active form, factor Xa, failed
to displace bound Fl-APC, suggesting that there is
a specific binding site for APC on the endothelial
15 cell surface. Protein C binding was dependent on
the Gla domain, since recombinant gla-domainless
protein C (rGDPC) failed to displace F1-APC.

        Detailed binding studies were also
performed with ^{125}I-labeled APC and monolayers of
20 HUVEC, as shown by Figures 2A, 2B, 2C and 2D. The
binding analysis indicated 7,000 sites per cell and
a Kd=30 nM. This affinity is similar to that
estimated from Figure 1.

        Endothelial cell surface thrombomodulin
25 can interact with protein C and APC. The Kd
(greater than 1 μM) (Hogg et al., (1992) J. Biol.
Chem. 267, 703-706; Olsen et al., (1992)
Biochemistry 31, 746-754), however, is much higher
than that of the binding site described above with
30 respect to the new receptor. Furthermore,
polyclonal and monoclonal antibodies against
thrombomodulin that inhibit protein C activation
did not inhibit the binding. Protein S also can
interact with protein C and APC (Dahlbäck et al.,
35 (1992) Biochemistry 31, 12769-12777), but F1-APC
binding to HUVEC was not influenced by protein S
addition. Furthermore, polyclonal and monoclonal

12

antibodies to protein S did not inhibit the binding. These results indicate the binding site for protein C and APC on endothelium is distinct from these known molecules.

5   **Nucleotide and Predicted Protein Structure Analysis of EPCR**

The insert was subcloned into pBluescript, and the nucleotide sequence was determined, as shown in Sequence ID No. 1. The

10 cDNA shown in Sequence ID No. 1 consists of 1302 bp, including a translation initiation ATG codon (AGGATGT, (Kozak, (1986) _Cell_ 44, 283-292) at the 5'-end at nucleotides 25-27 of Sequence ID No. 1. A potential polyadenylation signal sequence,

15 AATAAA, (Proudfoot and Brownlee, (1976) _Nature_ 263, 211-214) begins at nucleotide 1267 of Sequence ID No. 1, just 18 bp upstream of the poly(A) sequence.

The cDNA is predicted to code for a protein of 238 amino acids (Sequence ID No. 2),

20 which includes a 15 amino acid signal sequence (von Heijne, (1986) _Nucleic Acids Res._ 14, 4683-4690) at the N-terminal. Therefore, the mature protein is predicted to contain 223 amino acids. Sequence ID No. 2 is the predicted amino acid sequence of EPCR.

25 Amino acids 1-15 of Sequence ID No. 2 (MLTTLLPILLLSGWA) are the putative signal sequence determined by the method of von Heijne (von Heijne, 1986). Amino acids 211-236 of Sequence ID No. 2 (LVLGVLVGGFIIAGVAVGIFLCTGGR) are the putative

30 transmembrane domain. Potential N-glycosylation sites are present at amino acids 47-49, 64-66, 136-138, and 172-174 of Sequence ID No. 2. Extracellular cysteine residues are present at amino acids 17, 114, 118, and 186 of Sequence ID

35 No. 2. A potential transmembrane region (Engelman et al., (1986) _Annu. Rev. Biophys. Chem._ 15, 321-53) consisting of 23 amino acids was identified at

13

the C-terminal end (beginning at amino acid 216 of
Sequence ID No. 2).

The protein is predicted to be a type 1
transmembrane protein. The extracellular domain
5   contains four potential N-glycosylation sites and
four Cys residues. The cytoplasmic region contains
only three amino acids and terminates with a Cys,
which could be acylated to something or involved in
heterodimer formation with another peptide.
10   Although described with reference to
cloning and expression of the protein encoding
sequence, larger amounts of protein can be obtained
by expression in suitable recombinant host systems,
such as mammalian, yeast, bacteria, or insect
15 cells. Isolation can be facilitated by making
antibodies to the recombinant protein which are
then immobilized on substrates for use in
purification of additional receptors, as described
below.
20   As used herein, the nucleotide sequences
encoding the receptor include the sequence shown in
Sequence ID No. 1, and sequences having
conservative substitutions, additions or deletions
thereof which hybridize to Sequence ID No. 1 under
25 stringent conditions. As used herein, the amino
acids sequences constituting the receptor include
the sequence shown in Sequence ID No. 2, and
sequences having conservative substitutions,
additions or deletions thereof which form a
30 receptor having functionally equivalent biological
activity. It is well known to those skilled in the
art what constitutes conservative substitutions,
additions or deletions, and which could be readily
ascertained as encoding, or forming, a functionally
35 equivalent receptor molecule using the functional
assays described herein.

14

The hydropathic plot shown in Figures 4a-
4c was performed according to the method of Goldman
et al (Engelman et al., 1994) (solid line) and that
of Kyte and Doolittle (1982) J. Mol. Biol. 157,
5   105-132 (dotted line).

DNA and protein database searches
revealed that the sequence is related to the
centrosome-associated, cell cycle dependent murine
protein, CCD41, also referred as centrocyclin
10   (Rothbarth et al., (1993) J. Cell Sci. 104, 19-30),
as shown by Figures 5a-5b. The similarity in the
published sequence of murine CCD41 with human EPCR
led to the cloning and sequencing of the murine
EPCR. The sequence of murine EPCR is shown in
15   Figure 6. It is distinct from the published
sequence of CCD41.

The EPCR amino acid sequence was also
related to, but quite distinct from, the CD1/MHC
superfamily and the murine CD1.2, as also shown by
20   Figures 5a-5b. Based on the homology to CD1/MHC,
it is likely that EPCR contains two domains
consisting of residues 17-114 and 118-188. Of the
CD1 family members, CD1d is the most similar to
EPCR. In the mouse, CCD41 is associated
25   exclusively with the centrosome during G_1 but
becomes detectable elsewhere during the cell cycle,
reaching a maximum during G_2, except during the G_2/M
phase (Rothbarth et al., 1993). EPCR expression
appears restricted to endothelium, which would not
30   be expected for a cell cycle associated protein.

The identification of the protein C
receptor on endothelium suggests that the
endothelial cell binds protein C/APC through three
distinct mechanisms. In addition to EPCR, protein
35   S can bind APC/protein C on negatively charged
membrane surfaces that include the endothelium
(Stern et al., (1986) J. Biol. Chem. 261, 713-718),

but this is not cell type specific (Dahlbäck et al., 1992). Thrombomodulin in complex with thrombin can bind protein C and APC (Hogg et al., 1992). On endothelium, the protein S binding sites

5 (Nawroth and Stern, (1986) J. Exp. Med. 163, 740-745), thrombomodulin (Esmon, 1989) and EPCR are all down regulated by cytokines, indicating that inflammation can impair protein C pathway function at multiple levels.

10   The homology to the CD1/MHC family of proteins is especially interesting since it provides indications as to the function of EPCR. The CD1/MHC family has three extracellular domains termed α1,2 and 3. The extracellular domain of EPCR

15 contains four Cys residues that appear to correspond to two distinct domains. EPCR lacks the third domain of the CD1/MHC family, but the two domains have significant homology to the α1 and α2 domains of the CD1 protein family and the α2 domain

20 of the MHC class 1 protein, suggesting that these proteins evolved from a common ancestor. The first domain of EPCR, residues 17-114, contains two potential N glycosylation sites and is rich in ß strand structure, suggesting that it may form a ß

25 sheet. Despite the ß strand structure, consensus sequences (Williams and Barclay, (1988) Ann. Rev. Immunol. 6, 381-405) for the immunoglobulin superfamily of receptors are absent. The second domain of EPCR, residues 118-188, contains two

30 additional N glycosylation sites and, like the CD1/MHC family, this domain is predicted to have limited ß structure.

II: Modulation of Inflammation using EPCR.

          In vitro studies have suggested

35 anti-inflammatory activities for APC. For instance, an unusual carbohydrate sequence on protein C can inhibit inflammatory cell adhesion to

16

selectins (Grinnell, at al., (1994) Glycobiology, 4,
221-226). Modest inhibitory effects of APC have
been reported on TNF production (Grey et al.,
(1993) Transplant. Proc. 25, 2913-2914). EPCR

5   could contribute to these anti-inflammatory
mechanisms. Since the homologous protein family,
CD1, can be linked to CD8 (Ledbetter et al., (1985)
J. Immunol. 134, 4250-4254), it is also possible
that the proteins C receptor is linked to another

10   protein and signal through this second protein.
One of the CD1 family members, CD1d, has been
reported to promote T cell responses, possibly
involving binding to CD8 (Panja et al., (1993) J.
Exp. Med. 178, 1115-1119). CD1b has recently been

15   reported to serve as an antigen presenting molecule
(Porcelli et al., (1992) Nature 360, 593-597). The
ability to bind protein C/APC could then be linked
either directly or indirectly to signalling via
direct interaction with cells of the immune system.

20   Since the MHC class of proteins is involved in
presentation of proteins to cell receptors, the
concept of presentation of protein C/APC to
inflammatory cells as a means of elaborating
anti-inflammatory activity may also be involved.

25   This includes modulation of enzyme specificity such
as occurs with thrombin-thrombomodulin interaction
(Esmon, 1989). In this case, the EPCR-APC complex
might cleave biologically active peptides from
unknown substrates.

30   **EPCR mRNA Levels and APC Binding**
To determine the cellular specificity of
EPCR expression, the intensity of FL-APC binding to
HUVEC was compared to several human cell lines.
Fl-APC bound strongly only to HUVEC, and not to any

35   of the T, B, or monocytic cell lines tested. Cells
were incubated at room temperature without or with
160 nM Fl-APC in the presence of 1.3 mM $CaCl_2$.

17

Binding was analyzed by flow cytometry. Slight
binding was demonstrated with the osteosarcoma
line, HOS and the epidermoid carcinoma cell line,
HEp-2.

5   Total RNA was extracted from these cells
and hybridized with the EPCR cDNA probe for
Northern Blot Analysis. EPCR mRNA was detected by
Northern blot analysis for HUVEC, Jurkat, HEp-2,
Raji, HOS, and U937. Among the cells lines tested,
10   EPCR mRNA was detected at high levels only in
HUVEC. The calculated mRNA size of 1.3 kb was
identical to the size of the isolated cDNA. After
prolonged exposure, a weak signal was also detected
with the osteosarcoma cell line HOS and monocyte
15   cell line U937. Thus, both APC binding and EPCR
mRNA expression are very specific for endothelium.

**Effects of TNF on APC Binding and EPCR
MRNA Levels**

Several other members of the protein C
20   anticoagulant pathway are subject to regulation by
inflammatory cytokines (Esmon, 1989). For
instance, endothelial cell surface thrombomodulin
expression and message are known to be reduced by
exposure of the cells to TNF (Conway and Rosenberg,
25   1988; Lentz et al., 1991). To determine if a
similar process occurs with EPCR, HUVEC were
treated with TNF and APC binding and expression of
EPCR mRNA were examined. APC binding to HUVEC
decreased in a time dependent fashion. EPCR
30   activity decreased more rapidly than thrombomodulin
antigen. HUVEC were cultured for 0, 6, 24 and 48
hr, in the presence of TNF-α (10 ng/ml). Cells
were harvested and residual Fl-APC binding or
thrombomodulin (TM) expression was analyzed by flow
35   cytometry. Cell surface TM was stained with an
anti-TM murine monoclonal antibody and FITC--
conjugated anti-mouse IgG. The negative control is
without added fluorescent ligand.

HUVEC were treated with 10 ng/ml of TNF-α
for 0, 0.5, 1, 2, 3, 6, 10 and 24 hr, and message
was extracted and detected as described above. The
results demonstrated that the concentration of EPCR
5   mRNA was also reduced by TNF treatment. Message
levels and APC binding activity decreased in
parallel. Therefore, the TNF mediated
down-regulation of APC binding to endothelium
probably occurs at the level of mRNA expression.

10   Enhancement of inflammatory responses by
blocking binding of endogenous molecules to ECPCR
can be achieved by administration of compounds
binding to the receptor to a subject in need of
inhibition. The degree of binding is routinely
15 determined using assays such as those described
above. Compounds which are effective include
antibodies to the protein, fragments of antibodies
retaining the binding regions, and peptide
fragments of APC which include the Gla region.

20 Inhibition of the inflammatory response could be
obtained by infusing soluble EPCR. Alternatively,
localizing EPCR to surfaces in contact with blood
would render the surfaces anticoagulant by virtue
of the ability of EPCR to bind and concentrate the
25 anticoagulant APC at the surface. Alternatively,
the function of EPCR could be enhanced by
overexpressing the EPCR in endothelium used to coat
vascular grafts in patients with vascular disease
or on stents in cardiac patients.

30   The DNA sequence can also be used for
screening for other homologous or structurally
similar receptor proteins using hybridization
probes.

These methods and reagents and
35 pharmaceuticals are more readily understood by
reference to the following.

19

Screening of patient samples for expression of receptor proteins.

Patients with thrombosis or hyperinflammatory conditions could be screened for
5   defects in the EPCR gene. Sequence ID No. 1, and consecutive portions thereof of at least about seven nucleotides, more preferably fourteen to seventeen nucleotides, most preferably about twenty nucleotides, are useful in this screening using
10   hybridization assays of patient samples, including blood and tissues. Screening can also be accomplished using antibodies, typically labelled with a fluorescent, radiolabelled, or enzymatic label, or by isolation of target cells and
15   screening for binding activity as described in the examples above. Typically, one would screen for expression on either a qualitative or quantitative basis, and for expression of functional receptor. Labelling can be with ^{32}P, ^{35}S, fluorescein, biotin,
20   or other labels routinely used with methods known to those skilled in the art for labelling of proteins and/or nucleic acid sequences.

Hybridization Probes

Reaction conditions for hybridization of
25   an oligonucleotide probe or primer to a nucleic acid sequence vary from oligonucleotide to oligonucleotide, depending on factors such as oligonucleotide length, the number of G and C nucleotides, and the composition of the buffer
30   utilized in the hybridization reaction. Moderately stringent hybridization conditions are generally understood by those skilled in the art as conditions approximately 25°C below the melting temperature of a perfectly base-paired double-
35   stranded DNA. Higher specificity is generally achieved by employing incubation conditions having higher temperatures, in other words, more stringent conditions. In general, the longer the sequence

or higher the G and C content, the higher the
temperature and/or salt concentration required.
Chapter 11 of the well-known laboratory manual of
Sambrook et al., MOLECULAR CLONING: A LABORATORY MANUAL,
5   second edition, Cold-Spring Harbor Laboratory
Press, New York (1990) (which is incorporated by
reference herein), describes hybridization
conditions for oligonucleotide probes and primers
in great detail, including a description of the
10   factors involved and the level of stringency
necessary to guarantee hybridization with
specificity.

         The preferred size of a hybridization
probe is from 10 nucleotides to 100,000 nucleotides
15   in length. Below 10 nucleotides, hybridized
systems are not stable and will begin to denature
above 20°C. Above 100,000 nucleotides, one finds
that hybridization (renaturation) becomes a much
slower and incomplete process, as described in
20   greater detail in the text MOLECULAR GENETICS, Stent,
G.S. and R. Calender, pp. 213-219 (1971). Ideally,
the probe should be from 20 to 10,000 nucleotides.
Smaller nucleotide sequences (20-100) lend
themselves to production by automated organic
25   synthetic techniques. Sequences from 100-10,000
nucleotides can be obtained from appropriate
restriction endonuclease treatments. The labeling
of the smaller probes with the relatively bulky
chemiluminescent moieties may in some cases
30   interfere with the hybridization process.

                  *Generation of Antibodies for
                   Diagnostic or Therapeutic Use*
         Antibodies to the receptor proteins can
also be generated which are useful in detection,
35   characterization or isolation of receptor proteins,
as well as for modifying receptor protein activity,
in most cases, through inhibition of binding.
Antibodies are generated by standard techniques,

using human or animal receptor proteins.   Since the
proteins exhibit high evolutionary conservation, it
may be advantageous to generate antibodies to a
protein of a different species of origin than the
5   species in which the antibodies are to be tested or
utilized, looking for those antibodies which are
immunoreactive with the most evolutionarily
conserved regions. Antibodies are typically
generated by immunization of an animal using an
10   adjuvant such as Freund's adjuvant in combination
with an immunogenic amount of the protein
administered over a period of weeks in two to three
week intervals, then isolated from the serum, or
used to make hybridomas which express the
15   antibodies in culture. Because the methods for
immunizing animals yield antibody which is not of
human origin, the antibodies could elicit an
adverse effect if administered to humans. Methods
for "humanizing" antibodies, or generating less
20   immunogenic fragments of non-human antibodies, are
well known. A humanized antibody is one in which
only the antigen-recognized sites, or
complementarily-determining hypervariable regions
(CDRs) are of non-human origin, whereas all
25   framework regions (FR) of variable domains are
products of human genes. These "humanized"
antibodies present a lesser xenographic rejection
stimulus when introduced to a human recipient.
                    To accomplish humanization of a selected
30   mouse monoclonal antibody, the CDR grafting method
described by Daugherty, et al., (1991) <u>Nucl. Acids</u>
<u>Res.</u>, 19:2471-2476, incorporated herein by
reference, may be used. Briefly, the variable
region DNA of a selected animal recombinant anti-
35   idiotypic ScFv is sequenced by the method of
Clackson, T., et al., (1991) <u>Nature</u>, 352:624-688,
incorporated herein by reference. Using this

sequence, animal CDRs are distinguished from animal
framework regions (FR) based on locations of the
CDRs in known sequences of animal variable genes.
Kabat, H.A., et al., Sequences of Proteins of
5   Immunological Interest, 4th Ed. (U.S. Dept. Health
and Human Services, Bethesda, MD, 1987). Once the
animal CDRs and FR are identified, the CDRs are
grafted onto human heavy chain variable region
framework by the use of synthetic oligonucleotides
10   and polymerase chain reaction (PCR) recombination.
Codons for the animal heavy chain CDRs, as well as
the available human heavy chain variable region
framework, are built in four (each 100 bases long)
oligonucleotides. Using PCR, a grafted DNA
15   sequence of 400 bases is formed that encodes for
the recombinant animal CDR/human heavy chain FR
protection.
          The immunogenic stimulus presented by the
monoclonal antibodies so produced may be further
20   decreased by the use of Pharmacia's (Pharmacia LKB
Biotechnology, Sweden) "Recombinant Phage Antibody
System" (RPAS), which generates a single-chain Fv
fragment (ScFv) which incorporates the complete
antigen-binding domain of the antibody. In the
25   RPAS, antibody variable heavy and light chain genes
are separately amplified from the hybridoma mRNA
and cloned into an expression vector. The heavy
and light chain domains are co-expressed on the
same polypeptide chain after joining with a short
30   linker DNA which codes for a flexible peptide.
This assembly generates a single-chain Fv fragment
(ScFv) which incorporates the complete antigen-
binding domain of the antibody. Compared to the
intact monoclonal antibody, the recombinant ScFv
35   includes a considerably lower number of epitopes,
and thereby presents a much weaker immunogenic
stimulus when injected into humans.

23

The antibodies can be formulated in standard pharmaceutical carriers for administration to patients in need thereof. These include saline, phosphate buffered saline, and other aqueous

5   carriers, and liposomes, polymeric microspheres and other controlled release delivery devices, as are well known in the art. The antibodies can also be administered with adjuvant, such as muramyl dipeptide or other materials approved for use in

10   humans (Freund's adjuvant can be used for administration of antibody to animals).

<u>Screening for drugs modifying or altering
the extent of receptor function or
expression</u>

15   The receptor proteins are useful as targets for compounds which turn on, or off, or otherwise regulate binding to these receptors. The assays described above clearly provide routine methodology by which a compound can be tested for

20   an inhibitory effect on binding of PC or APC. The *in vitro* studies of compounds which appear to inhibit binding selectively to the receptors are then confirmed by animal testing. Since the molecules are so highly evolutionarily conserved,

25   it is possible to conduct studies in laboratory animals such as mice to predict the effects in humans.

In cases where inflammatory mediators or vascular disease down regulate EPCR, it would be

30   advantageous to increase its concentration *in vivo* on endothelium. The binding assays described here and the gene sequence allow assays for increased EPCR expression. Similar approaches have been taken with thrombomodulin and investigators have

35   shown that cyclic AMP (Maruyama,I. et al. (1991) <u>Thrombosis Research</u> 61, 301-310) and interleukin 4 (Kapiotis,S. et al., (1991) <u>Blood</u> 78, 410-415) can elevate thrombomodulin expression. The ability to

24

screen such drugs or drugs that block TNF down
regulation of EPCR provide an approach to elevating
EPCR expression in vivo and thus enhancing
anticoagulant, and anti-inflammatory activity.

5   Studies based on inhibition of binding
are predictive for indirect effects of alteration
of receptor binding. For example, inhibition of
binding of APC or increased expression of TNF is
predictive of inhibition of EPCR function.

10   Assays for testing compounds for useful
activity can be based solely on interaction with
the receptor protein, preferably expressed on the
surface of transfected cells such as those
described above. Proteins in solution or
15   immobilized on inert substrates can also be
utilized. These can be used to detect inhibition
or enhancement in binding of PC or APC
          Alternatively, the assays can be based on
interaction with the gene sequence encoding the
20   receptor protein, preferably the regulatory
sequences directing expression of the receptor
protein. For example, antisense which binds to the
regulatory sequences, and/or to the protein
encoding sequences can be synthesized using
25   standard oligonucleotide synthetic chemistry. The
antisense can be stabilized for pharmaceutical use
using standard methodology (encapsulation in a
liposome or microsphere; introduction of modified
nucleotides that are resistant to degradation or
30   groups which increase resistance to endonucleases,
such as phosphorothiodates and methylation), then
screened initially for alteration of receptor
activity in transfected or naturally occurring
cells which express the receptor, then in vivo in
35   laboratory animals. Typically, the antisense would
inhibit expression. However, sequences which block

those sequences which "turn off" synthesis can also
be targeted.

The receptor protein for study can be
isolated from either naturally occurring cells or
5   cells which have been genetically engineered to
express the receptor, as described in the examples
above. In the preferred embodiment, the cells
would have been engineered using the intact gene.

                    Random generation of receptor or
10   *receptor encoding sequence binding*
                    molecules.
        Molecules with a given function,
catalytic or ligand-binding, can be selected for
from a complex mixture of random molecules in what
15   has been referred to as "in vitro genetics"
(Szostak, (1992) TIBS 19:89). One synthesizes a
large pool of molecules bearing random and defined
sequences and subjects that complex mixture, for
example, approximately 10^{15} individual sequences in
20   100 μg of a 100 nucleotide RNA, to some selection
and enrichment process. For example, by repeated
cycles of affinity chromatography and PCR
amplification of the molecules bound to the ligand
on the column, Ellington and Szostak (1990)
25   estimated that 1 in 10^{10} RNA molecules folded in
such a way as to bind a given ligand. DNA
molecules with such ligand-binding behavior have
been isolated (Ellington and Szostak, 1992; Bock et
al, 1992).
30   *Computer assisted drug design*
        Computer modeling technology allows
visualization of the three-dimensional atomic
structure of a selected molecule and the rational
design of new compounds that will interact with the
35   molecule. The three-dimensional construct
typically depends on data from x-ray
crystallographic analyses or NMR imaging of the
selected molecule. The molecular dynamics require

26

force field data. The computer graphics systems
enable prediction of how a new compound will link
to the target molecule and allow experimental
manipulation of the structures of the compound and
5   target molecule to perfect binding specificity.
Prediction of what the molecule-compound
interaction will be when small changes are made in
one or both requires molecular mechanics software
and computationally intensive computers, usually
10   coupled with user-friendly, menu-driven interfaces
between the molecular design program and the user.
Examples of molecular modelling systems
are the CHARMm and QUANTA programs, Polygen
Corporation, Waltham, MA. CHARMm performs the
15.   energy minimization and molecular dynamics
functions. QUANTA performs the construction,
graphic modelling and analysis of molecular
structure. QUANTA allows interactive construction,
modification, visualization, and analysis of the
20   behavior of molecules with each other.
A number of articles review computer
modeling of drugs interactive with specific
proteins, such as Rotivinen, et al., (1988) _Acta_
Pharmaceutica Fennica 97, 159-166; Ripka, _New_
25   _Scientist_ 54-57 (June 16, 1988); McKinaly and
Rossmann, (1989) _Annu. Rev. Pharmacol. Toxiciol._
29, 111-122; Perry and Davies, _QSAR: Quantitative_
Structure-Activity Relationships in Drug Design pp.
189-193 (Alan R. Liss, Inc. 1989); Lewis and Dean,
30   (1989) _Proc. R. Soc. Lond._ 236, 125-140 and 141-
162; and, with respect to a model receptor for
nucleic acid components, Askew, et al., (1989) _J._
Am. Chem. Soc. 111, 1082-1090. Other computer
programs that screen and graphically depict
35   chemicals are available from companies such as
BioDesign, Inc., Pasadena, CA., Allelix, Inc,
Mississauga, Ontario, Canada, and Hypercube, Inc.,

27

Cambridge, Ontario. Although these are primarily designed for application to drugs specific to particular proteins, they can be adapted to design of drugs specific to regions of DNA or RNA, once

5   that region is identified.

Although described above with reference to design and generation of compounds which could alter binding, one could also screen libraries of known compounds, including natural products or

10   synthetic chemicals, and biologically active materials, including proteins, for compounds which are inhibitors or activators.

Generation of nucleic acid regulators

Nucleic acid molecules containing the 5'

15   regulatory sequences of the receptor genes can be used to regulate or inhibit gene expression *in vivo*. Vectors, including both plasmid and eukaryotic viral vectors, may be used to express a particular recombinant 5' flanking region-gene

20   construct in cells depending on the preference and judgment of the skilled practitioner (see, e.g., Sambrook et al., Chapter 16). Furthermore, a number of viral and nonviral vectors are being developed that enable the introduction of nucleic

25   acid sequences *in vivo* (see, e.g., Mulligan, (1993) Science, 260, 926-932; United States Patent No. 4,980,286; United States Patent No. 4,868,116; incorporated herein by reference). Recently, a delivery system was developed in which nucleic acid

30   is encapsulated in cationic liposomes which can be injected intravenously into a mammal. This system has been used to introduce DNA into the cells of multiple tissues of adult mice, including endothelium and bone marrow (see, e.g., Zhu et al.,

35   (1993) Science 261, 209-211; incorporated herein by reference).

28

The 5' flanking sequences of the receptor
gene can also be used to inhibit th expression of
the receptor. For example, an antisense RNA of all
or a portion of the 5' flanking region of the

5   receptor gene can be used to inhibit expression of
the receptor *in vivo*. Expression vectors (e.g.,
retroviral expression vectors) are already
available in the art which can be used to generate
an antisense RNA of a selected DNA sequence which

10   is expressed in a cell (see, e.g., U.S. Patent No.
4,868,116; U.S. Patent No. 4,980,286).
Accordingly, DNA containing all or a portion of the
sequence of the 5' flanking region of the receptor
gene can be inserted into an appropriate expression

15   vector so that upon passage into the cell, the
transcription of the inserted DNA yields an
antisense RNA that is complementary to the mRNA
transcript of the receptor protein gene normally
found in the cell. This antisense RNA transcript

20   of the inserted DNA can then base-pair with the
normal mRNA transcript found in the cell and
thereby prevent the mRNA from being translated. It
is of course necessary to select sequences of the
5' flanking region that are downstream from the

25   transcriptional start sites for the receptor
protein gene to ensure that the antisense RNA
contains complementary sequences present on the
mRNA.

Antisense RNA can be generated *in vitro*
30   also, and then inserted into cells.
Oligonucleotides can be synthesized on an automated
synthesizer (e.g., Model 8700 automated synthesizer
of Milligen-Biosearch, Burlington, MA or ABI Model
380B). In addition, antisense

35   deoxyoligonucleotides have been shown to be
effective in inhibiting gene transcription and
viral replication (see e.g., Zamecnik et al.,

(1978) Proc. Natl. Acad. Sci. USA 75, 280-284;
Zamecnik et al., (1986) Proc. Natl. Acad. Sci., 83,
4143-4146; Wickstrom et al. (1988) Proc. Natl.
Acad. Sci. USA 85, 1028-1032; Crooke, (1993) FASEB
5   J. 7, 533-539. Furthermore, recent work has shown
that improved inhibition of expression of a gene by
antisense oligonucleotides is possible if the
antisense oligonucleotides contain modified
nucleotides (see, e.g., Offensperger et al.,
10   (1993) EMBO J. 12, 1257-1262 (in vivo inhibition of
duck hepatitis B viral replication and gene
expression by antisense phosphorothioate
oligodeoxynucleotides); PCT WO 93/01286 Rosenberg
et al., (synthesis of sulfurthioate
15   oligonucleotides); Agrawal et al., (1988) Proc.
Natl. Acad. Sci. USA 85, 7079-7083 (synthesis of
antisense oligonucleoside phosphoramidates and
phosphorothioates to inhibit replication of human
immunodeficiency virus-1); Sarin et al., (1989)
20   Proc. Natl. Acad. Sci. USA 85, 7448-7794 (synthesis
of antisense methylphosphonate oligonucleotides);
Shaw et al., (1991) Nucleic Acids Res 19, 747-750
(synthesis of 3' exonuclease-resistant
oligonucleotides containing 3' terminal
25   phosphoroamidate modifications); incorporated
herein by reference).
The sequences of the 5' flanking region
of receptor protein gene can also be used in triple
helix (triplex) gene therapy. Oligonucleotides
30   complementary to gene promoter sequences on one of
the strands of the DNA have been shown to bind
promoter and regulatory sequences to form local
triple nucleic acid helices which block
transcription of the gene (see, e.g., Maher et al.,
35   (1989) Science 245, 725-730; Orson et al., (1991)
Nucl. Acids Res. 19, 3435-3441; Postal et al.,
(1991) Proc. Natl. Acad. Sci. USA 88, 8227-8231;

Cooney et al., (1988) Science 241, 456-459; Young
et al., (1991) Proc. Natl. Acad. Sci. USA 88,
10023-10026; Duval-Valentin et al., (1992) Proc.
Natl. Acad. Sci. USA 89, 504-508; Blume et al.,
(1992) Nucl. Acids Res. 20, 1777-1784; Grigoriev et
al., (1992) J. Biol. Chem. 267, 3389-3395.

Recently, both theoretical calculations
and empirical findings have been reported which
provide guidance for the design of oligonucleotides
for use in oligonucleotide-directed triple helix
formation to inhibit gene expression. For example,
oligonucleotides should generally be greater than
14 nucleotides in length to ensure target sequence
specificity (see, e.g., Maher et al., (1989);
Grigoriev et al., (1992)). Also, many cells avidly
take up oligonucleotides that are less than 50
nucleotides in length (see e.g., Orson et al.,
(1991); Holt et al., (1988) Mol. Cell. Biol. 8,
963-973; Wickstrom et al., (1988) Proc. Natl. Acad.
Sci. USA 85, 1028-1032). To reduce susceptibility
to intracellular degradation, for example by 3'
exonucleases, a free amine can be introduced to a
3' terminal hydroxyl group of oligonucleotides
without loss of sequence-binding specificity (Orson
et al., 1991). Furthermore, more stable triplexes
are formed if any cytosines that may be present in
the oligonucleotide are methylated, and also if an
intercalating agent, such as an acridine
derivative, is covalently attached to a 5' terminal
phosphate (e.g., via a pentamethylene bridge);
again without loss of sequence specificity (Maher
et al., (1989); Grigoriev et al., (1992).

Methods to produce or synthesize
oligonucleotides are well known in the art. Such
methods can range from standard enzymatic digestion
followed by nucleotide fragment isolation (see
e.g., Sambrook et al., Chapters 5, 6) to purely

synthetic methods, for example, by the cyanoethyl
phosphoramidite method using a Milligen or Beckman
System 1Plus DNA synthesizer (see also, Ikuta et
al., (1984) Ann. Rev. Biochem. 53, 323-356

5   (phosphotriester and phosphite-triester methods);
Narang et al., (1980) Methods Enzymol., 65, 610-620
(phosphotriester method). Accordingly, DNA
sequences of the 5' flanking region of the receptor
protein gene described herein can be used to design

10 and construct oligonucleotides including a DNA
sequence consisting essentially of at least 15
consecutive nucleotides, with or without base
modifications or intercalating agent derivatives,
for use in forming triple helices specifically

15 within the 5' flanking region of a receptor protein
gene in order to inhibit expression of the gene.
          In some cases it may be advantageous to
insert enhancers or multiple copies of the
regulatory sequences into an expression system to

20 facilitate screening of methods and reagents for
manipulation of expression.
          Preparation of Receptor Protein Fragments
          Compounds which are effective for
blocking binding of the receptor can also consist

25 of fragments of the receptor proteins, expressed
recombinantly and cleaved by enzymatic digest or
expressed from a sequence encoding a peptide of
less than the full length receptor protein. These
will typically be soluble proteins, i.e., not

30 including the transmembrane and cytoplasmic
regions, although smaller portions determined in
the assays described above to inhibit or compete
for binding to the receptor proteins can also be
utilized. It is a routine matter to make

35 appropriate receptor protein fragments, test for
binding, and then utilize. The preferred fragm nts
are of human origin, in order to minimize potential

32

immunological response. The peptides can be as
short as five to eight amino acids in length and
are easily prepared by standard techniques. They
can also be modified to increase *in vivo* half-life,
5　by chemical modification of the amino acids or by
attachment to a carrier molecule or inert
substrate. Based on studies with other peptide
fragments blocking receptor binding, the IC_{50}, the
dose of peptide required to inhibit binding by 50%,
10　ranges from about 1 μM to greater than 10 mM,
depending on the peptide size and folding. These
ranges are well within the effective concentrations
for the *in vivo* administration of peptides, based
on comparison with the RGD-containing peptides,
15　described, for example, in U.S. Patent No.
4,792,525 to Ruoslaghti, et. al., used *in vivo* to
alter cell attachment and phagocytosis. The
peptides can also be conjugated to a carrier
protein such as keyhole limpet hemocyanin by its N-
20　terminal cysteine by standard procedures such as
the commercial Imject kit from Pierce Chemicals or
expressed as a fusion protein, which may have
increased efficacy.

As noted above, the peptides can be
25　prepared by proteolytic cleavage of the receptor
proteins, or, preferably, by synthetic means.
These methods are known to those skilled in the
art. An example is the solid phase synthesis
described by J. Merrifield, (1964) J. Am. Chem.
30　Soc. 85, 2149, used in U.S. Patent No. 4,792,525,
and described in U.S. Patent No. 4,244,946, wherein
a protected alpha-amino acid is coupled to a
suitable resin, to initiate synthesis of a peptide
starting from the C-terminus of the peptide. Other
35　methods of synthesis are described in U.S. Patent
No. 4,305,872 and 4,316,891. These methods can be
used to synthesize peptides having identical

33

sequence to the receptor proteins described herein, or substitutions or additions of amino acids, which can be screened for activity as described above.

The peptide can also be administered as a
5   pharmaceutically acceptable acid- or base- addition salt, formed by reaction with inorganic acids such as hydrochloric acid, hydrobromic acid, perchloric acid, nitric acid, thiocyanic acid, sulfuric acid, and phosphoric acid, and organic acids such as
10   formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, malonic acid, succinic acid, maleic acid, and fumaric acid, or by reaction with an inorganic base such as sodium hydroxide, ammonium hydroxide,
15   potassium hydroxide, and organic bases such as mono-, di-, trialkyl and aryl amines and substituted ethanolamines.

Peptides containing cyclopropyl amino acids, or amino acids derivatized in a similar
20   fashion, can also be used. These peptides retain their original activity but have increased half-lives *in vivo*. Methods known for modifying amino acids, and their use, are known to those skilled in the art, for example, as described in U.S. Patent
25   No. 4,629,784 to Stammer.

The peptides are generally active when administered parenterally in amounts above about 1 μg/kg of body weight. Based on extrapolation from other proteins, for treatment of most inflammatory
30   disorders, the dosage range will be between 0.1 to 70 mg/kg of body weight. This dosage will be dependent, in part, on whether one or more peptides are administered.

Pharmaceutical Compositions
35   Compounds which alter receptor protein binding are preferably administered in a pharmaceutically acceptable vehicle. Suitable

34

pharmaceutical vehicles are known to those skilled
in the art. For parenteral administration, the
compound will usually be dissolved or suspended in
sterile water or saline. For enteral

5   administration, the compound will be incorporated
into an inert carrier in tablet, liquid, or
capsular form. Suitable carriers may be starches
or sugars and include lubricants, flavorings,
binders, and other materials of the same nature.

10 The compounds can also be administered locally by
topical application of a solution, cream, gel, or
polymeric material (for example, a Pluronic™,
BASF).

Alternatively, the compound may be

15 administered in liposomes or microspheres (or
microparticles). Methods for preparing liposomes
and microspheres for administration to a patient
are known to those skilled in the art. U.S. Patent
No. 4,789,734 describe methods for encapsulating

20 biological materials in liposomes. Essentially,
the material is dissolved in an aqueous solution,
the appropriate phospholipids and lipids added,
along with surfactants if required, and the
material dialyzed or sonicated, as necessary. A

25 review of known methods is by G. Gregoriadis,
Chapter 14. "Liposomes", Drug Carriers in Biology
and Medicine pp. 287-341 (Academic Press, 1979).
Microspheres formed of polymers or proteins are
well known to those skilled in the art, and can be

30 tailored for passage through the gastrointestinal
tract directly into the bloodstream.
Alternatively, the compound can be incorporated and
the microspheres, or composite of microspheres,
implanted for slow release over a period of time,

35 ranging from days to months. See, for example,
U.S. Patent Nos. 4,906,474, 4,925,673, and
3,625,214.

35

Disorders to be treated

As described herein, a variety of
compounds can be used to inhibit or enhance
expression of the EPCR. The nature of the disorder
5   will determine if the expression should be enhanced
or inhibited. For example, based on the studies
involving the use of an anti-protein C antibody in
combination with cytokine, it should be possible to
treat solid tumors by enhancing an inflammatory
10   response involving blocking of protein C or
activated protein C binding to an endothelial cell
protein C/activated protein C receptor by
administering to a patient in need of treatment
thereof an amount of a compound blocking binding of
15   protein C or activated protein C to the receptor.
Similarly, it should be possible to treat disorders
such as gram negative sepsis, stroke, thrombosis,
septic shock, adult respiratory distress syndrome,
and pulmonary emboli using a method for inhibiting
20   an inflammatory response involving administration
of EPCR or EPCR fragments or substances that
upregulate EPCR expression to a patient in need of
treatment thereof.

SEQUENCE LISTING

(1) GENERAL INFORMATION:
        (i) APPLICANT: Oklahoma Medical Research Foundation
       (ii) TITLE OF INVENTION: Cloning and Regulation of an Endothelial Cell Protein
C/Activated Protein C Receptor
      (iii) NUMBER OF SEQUENCES: 2
       (iv) CORRESPONDENCE ADDRESS:
            (A) ADDRESSEE: Patrea L. Pabst
            (B) STREET: 2800 One Atlantic Center
                1201 West Peachtree Street
            (C) CITY: Atlanta
            (D) STATE: Georgia
            (E) COUNTRY: USA
            (F) ZIP: 30309-3450
        (v) COMPUTER READABLE FORM:
            (A) MEDIUM TYPE: Floppy disk
            (B) COMPUTER: IBM PC compatible
            (C) OPERATING SYSTEM: PC-DOS/MS-DOS
            (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
     (viii) ATTORNEY/AGENT INFORMATION:
            (A) NAME: Pabst, Patrea L.
            (B) REGISTRATION NUMBER: 31,284
            (C) REFERENCE/DOCKET NUMBER: OMRF152
       (ix) TELECOMMUNICATION INFORMATION:
            (A) TELEPHONE: (404) 873-8794
            (B) TELEFAX: (404) 873-8795

(2) INFORMATION FOR SEQ ID NO:1:
        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 1302 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear
       (ii) MOLECULE TYPE: cDNA
      (iii) HYPOTHETICAL: NO
       (iv) ANTI-SENSE: NO
       (ix) FEATURE:
            (A) NAME/KEY: misc_feature

```
(B) LOCATION: 1..1302
(D) OTHER INFORMATION: /note= "Nucleotides 25 through 738 encode the
Endothelial Cell Protein Receptor of Sequence ID No. 2."
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:
```

```
CAGGTCCGGA GCCTCAACTT CAGGATGTTG ACAACATTGC TGCCGATACT GCTGCTGTCT      60

GGCTGGGCCT TTTGTAGCCA AGACGCCTCA GATGGCCTCC AAAGACTTCA TATGCTCCAG      120

ATCTCCTACT TCCGCGACCC CTATCACGTG TGGTACCAGG GCAACGGCGTC GCTGGGGGGA      180

CACCTAACGC ACGTGCTGGA AGGCCCAGAC ACCAACACCA CGATCATTCA GCTGCAGCCC      240

TTGCAGGAGC CCGAGAGCTG GGCGCGCACG CAGAGTGCC TGCAGTCCTA CCTGCTCCAG       300

TTCCACGGCC TCGTGCGCCT GGTGCACCAG GAGCGGACCT TGGCCTTTCC TCTGACCATC      360

CGCTGCTTCC TGGGCTGTGA GCTGCCTCCC GAGGGCTCTA GAGCCCATGT CTTCTTCGAA      420

GTGGCTGTGA ATGGGAGCTC CTTTGTGAGT TTCCGGCCGG AGAGAGCCTT GTGGCAGGCA      480

GACACCCAGG TCACCTCCGG AGTGGTCACC TTCACCCTGC AGCAGCTCAA TGCCTACAAC      540

CGCACTCGGT ATGAACTGCG GGAATTCCTG GAGGACACCT GTGTGCAGTA TGTGCAGAAA      600

CATATTTCCG CGGAAAACAC GAAAGGGAGC CAAACAAGCC GCTCCTACAC TTCGCTGGTC      660

CTGGGGCGTCC TGGTGGGCGG TTTCATCATT GCTGGTGTGG CTGTAGGCAT CTTCCTGTGC      720

ACAGGTGGAC GGCGATGTTA ATTACTCTCC AGCCCCGTCA GAAGGGGCTG GATTGATGGA      780

GGCTGGCAAG GGAAAGTTTC AGCTCACTGT GAAGCCAGAC TCCCAACTG AAACACCAGA       840

AGGTTTGGAG TGACAGCTCC TTTCTTCTCC CACATCTGCC CACTGAAGAT TTGAGGGAGG      900

GGAGATGGAG AGGAGAGGTG GACAAAGTAC TTGGTTTGCT AAGAACCTAA GAACGTGTAT      960
```
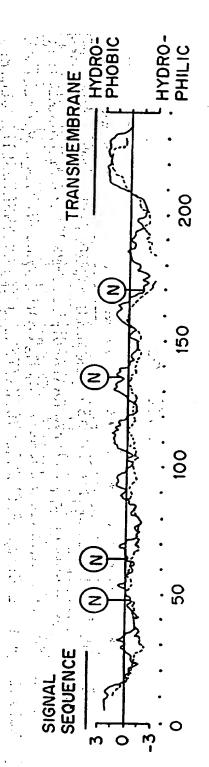
```
GCTTGCTGA ATTAGTCTGA TAAGTGAATG TTTATCTATC TTTGTGAAAA ACAGATAATG    1020

GAGTTGGGGC AGGAAGCCTA TGCGCCATCC TCCAAAGACA GACAGAATCA CCTGAGGCGT    1080

TCAAAAGATA TAACCAAATA AACAAGTCAT CCACAATCAA AATACAACAT TCAATACTTC    1140

CAGGTGTGTC AGACTTGGGA TGGGACGCTG ATATAAATAGG GTAGAAAGAA GTAACACGAA    1200

GAAGTGGTGG AAATGTAAAA TCCAAGTCAT ATGGCAGTGA TCAATTATTA ATCAATTAAT    1260

AATATTAATA AATTCTTAT ATTTAAAAAA AAAAAAAAA AA                         1302
```

(2) INFORMATION FOR SEQ ID NO:2:

   (i) SEQUENCE CHARACTERISTICS:

      (A) LENGTH: 238 amino acids

      (B) TYPE: amino acid

      (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: protein

  (iii) HYPOTHETICAL: NO

   (ix) FEATURE:

      (A) NAME/KEY: misc_feature

      (B) LOCATION: 1..365

      (D) OTHER INFORMATION: /note= "Endothelial Cell Protein Receptor encoded by nucleotides 1 through 1302 of Sequence ID No. 1."

   (ix) FEATURE:

      (A) NAME/KEY: Modified-site

      (B) LOCATION: 1..15

      (D) OTHER INFORMATION: /note= "Amino acids 1-15 represent a putative signal sequence."

   (ix) FEATURE:

      (A) NAME/KEY: Domain

      (B) LOCATION: 211..236

      (D) OTHER INFORMATION: /note= "Amino acids 211-236 represent a putative transmembrane domain."

   (ix) FEATURE:

      (A) NAME/KEY: Activ -site

      (B) LOCATION: 47..174

(D) OTHER INFORMATION: /note= "Amino acids 47-49, 64-66,
    136-138 and 172-174 represent potential
    N-glycosylation sites."

(ix) FEATURE:
    (A) NAME/KEY: Modified-site
    (B) LOCATION: 17..186
    (D) OTHER INFORMATION: /note= "Amino acids 17, 114, 118
        and 186 represent extracellular cysteine
        residues."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Leu Thr Thr Leu Leu Pro Ile Leu Leu Ser Gly Trp Ala Phe
1               5                   10                  15

Cys Ser Gln Asp Ala Ser Asp Gly Leu Gln Arg Leu His Met Leu Gln
                20                  25                  30

Ile Ser Tyr Phe Arg Asp Pro Tyr His Val Trp Tyr Gln Gly Asn Ala
            35                  40                  45

Ser Leu Gly Gly His Leu Thr His Val Leu Glu Gly Pro Asp Thr Asn
50                  55                  60

Thr Thr Ile Ile Gln Leu Gln Pro Leu Gln Gln Glu Pro Glu Ser Trp Ala
65                  70                  75                  80

Arg Thr Gln Ser Gly Leu Gln Ser Tyr Leu Leu Gln Phe His Gly Leu
                85                  90                  95

Val Arg Leu Val His Gln Glu Arg Thr Leu Ala Phe Pro Leu Thr Ile
                100                 105                 110

Arg Cys Phe Leu Gly Cys Glu Leu Pro Pro Glu Gly Ser Arg Ala His
                115                 120                 125

Val Phe Phe Glu Val Ala Val Asn Gly Ser Ser Phe Val Ser Phe Arg
                130                 135                 140
```

40

Pro Glu Arg Ala Leu Trp Gln Ala Asp Thr Gln Val Thr Ser Gly Val
145   150   155   160

Val Thr Phe Thr Leu Gln Gln Leu Asn Ala Tyr Asn Arg Thr Arg Tyr
                165   170   175

Glu Leu Arg Glu Phe Leu Glu Asp Thr Cys Val Gln Tyr Val Gln Lys
                180   185   190

His Ile Ser Ala Glu Asn Thr Lys Gly Ser Gln Thr Ser Arg Ser Tyr
                195   200   205

Thr Ser Leu Val Leu Gly Val Leu Ile Gly Gly Phe Ile Ile Ala Gly
210   215   220

Val Ala Val Gly Ile Phe Leu Cys Thr Gly Gly Arg Arg Cys
225   230   235

We claim.

1.   An isolated endothelial cell protein
C/activated protein C receptor.

2.   The receptor of claim 1 encoded by
the nucleotide sequence of Sequence ID No. 1 and
degenerative sequences thereof and sequence having
conservative substitutions, additions or deletions
thereof hybridizing to Sequence ID No. 1 under
stringent conditions, which encode the receptor.

3.   The receptor of claim 1 having the
amino acid sequence of Sequence ID No. 2 or a
sequence having conservative substitutions,
additions or deletions thereof.

4.   The receptor of claim 1 expressed on
the surface of a non-human cell or a non-
endothelial cell.

5.   The receptor of claim 1 in soluble
form.

6.   The receptor of claim 5 lacking at
least a portion of the transmembrane region.

7.   A nucleotide sequence encoding an
endothelial cell protein C/activated protein C
receptor.

8.   The sequence of claim 7 having the
nucleotide sequence of Sequence ID No. 1 or
degenerative sequences thereof and sequence having
conservative substitutions, additions or deletions
thereof and hybridizing under stringent conditions
to Sequence ID No. 1.

9.   The sequence of claim 7 encoding the
amino acid sequence of Sequence ID No. 2 or a
sequence having conservative substitutions,
additions or deletions thereof.

10.   The sequence of claim 7 further
comprising an expression vector.

11.   The sequence of claim 10 further
comprising an expression host.

12. The sequence of claim 10 expressed
on the surface of a non-human cell or a non-
endothelial cell.

13. The sequence of claim 7 encoding a
soluble form of the receptor.

14. The sequence of claim 7 encoding a
fragment of the receptor of at least fourteen
consecutive nucleotides in length.

15. The sequence of claim 14 labelled
with a detectable label.

16. A method for enhancing an
inflammatory response involving blocking of protein
C or activated protein C binding to an endothelial
cell protein C/activated protein C receptor
comprising administering to a patient in need of
treatment thereof an amount of a compound blocking
binding of protein C or activated protein C to the
receptor. tumors

17. The method of claim 16 wherein the
compound is selected from the group consisting of
antibodies and fragments of antibodies to the
receptor, nucleic acid sequences inhibiting
expression of the receptor, and synthetic or
natural compounds other than proteins, peptides or
nucleic acid sequences which inhibit binding.

18. A method for inhibiting an
inflammatory response involving administration of a
compound selected from the group consisting of EPCR
or EPCR fragments and substances that upregulate
EPCR expression to a patient in need of treatment
thereof.

19. An antibody or antibody fragment
specifically immunoreactive with a unique epitope
of an isolated endothelial cell protein C/activated
protein C receptor.

20. The antibody of claim 19 wherein the
receptor is encoded by the nucleotide sequence of

43

Sequence ID No. 1 and degenerative sequences
thereof and sequence having conservative
substitutions, additions or deletions thereof and
hybridizing to Sequence ID No. 1 under stringent
conditions.

21. The antibody of claim 19 wherein the
receptor has the amino acid sequence of Sequence ID
No. 2 or a sequence having conservative
substitutions, additions or deletions thereof.

22. A method for screening for a
compound which alters the binding of an endothelial
receptor protein to protein C or activated protein
C comprising   providing an assay for binding of
protein C or activated protein C to the receptor
protein,

adding the compound to be tested to the
assay, and

determining if the amount of protein C or
activated protein C which is bound to the receptor
protein is altered as compared to binding in the
absence of the compound to be tested.

23. A method for screening patients for
abnormal receptor protein activity or function
comprising

determining the presence of an
endothelial cell surface receptor binding protein C
and activated protein C, and

comparing the receptor to determine if
the quantity present or the function of the
receptor is equivalent to that present in normal
cells.

FIG. 1a

FIG. 1b

FIG. 1c

SUBSTITUTE SHEET (RULE 26)

2/10

FIG. 2a

FIG. 2b

FIG. 2c

FIG. 2d

FIG. 3a

FIG. 3b

FIG. 4a

```
CAGGTCCGGAGCCTCAACTTCAGGATGTTGACAACATTGCTGCCGATACTGCTGTGTCT   60
                          M  L  T  T  L  L  P  I  L  L  S         12

GGCTGGGCCTTTGTAGCCAAGACGCCTCAGATGGCCTCAGATGGCCTCCAAAGACTTCATATGCTCCAG  120
G  W  A  F  C  S  Q  D  A  S  D  G  L  Q  R  L  H  M  L  Q      32

ATCTCCTACTTCCGGCGACCCCTATCACGTGTGGTACCAGGGCAACGCG TCGCTGGGGGGA  180
I  S  Y  F  R  D  P  Y  H  V  W  Y  Q  G  [N  A  S] L  G  G    52

CACCTAAACGCACGTGCTGGAAGGCCCAGACAACACCACGATCATTCAGCTGCAGCCC   240
H  L  T  H  V  L  E  G  P  D  [T  N  T] I  I  Q  L  Q  P      72

TTGCAGGAGCCCGAGAGCTGGGCGCCACGCGGCCTGCAGTCCTACCTGCTCCAG      300
L  Q  E  P  E  S  W  A  R  T  Q  S  G  L  Q  S  Y  L  L  Q    92

TTCCACGGCCTCGTGCGCCTGGTTGCACCAGGAGCGGGACCTTGGCCTTCCTCTGACCATC  360
F  H  G  L  V  R  L  V  H  Q  E  R  T  L  A  F  P  L  T  I     112

CGCTGCTTCCTGGGCTGTGAGCTGCCCCCGGAGCCCATGTCTTCTTCGAA         420
R  C  F  L  G  C  E  L  P  P  E  G  S  R  A  H  V  F  F  E    132
```

SUBSTITUTE SHEET (RULE 26)

FIG. 4b

GTGGCTGTGAATGGGAGCTCCTTTGTGAGTTCCGGCCGGAGAGAGCCTTGTGGCAGGCA 480
V A V N G S S F V S F R P E R A L W Q A   152

GACACCCAGGTCACCTCCGGAGTGGTCACCTTCACCCTGCAGCAGCTCAATGCCTACAAC 540
D T Q V T S G V V T F T L Q Q L N A Y N   172

CGCACTCGGTATGAACTGCGGGAATTCCTGGAGGACACCTGTGTGCAGTATGTGCAGAAA 600
R T R Y E L R E F L E D T C V Q Y V Q K   192

CATATTTCCCGGGAAAACACGAAAGGGAGCCAAACAGCCGCTCCTACACTTCGGCTGGTC 660
H I S A E N T K G S Q T S R S Y T S L V   212

CTGGGCGTCCTGGTGTGGGCGGGTTCATCATTGCTGGTGTGGCTGTAGGCATCTTCCTGTGC 720
L G V L V G G F I I A G V A V G I F L C   232

ACAGGTGGACGGCGATGTTAATTACTCTCCAGCCCCGTCAGAAGGGGCTGGATTGATGGA 780
T G R R C *   238

SUBSTITUTE SHEET (RULE 26)

FIG. 4C

```
GGCTGGGCAAGGGAAAGTTTCAGCTCACTGTGAAGCCAGACTCCCAACTGAAACACCAGA    840
AGGTTTGGAGTGACAGCTCCTTCTTCTCCCACATCTGCCCACTGAAGATTGAGGGAGG     900
GGAGATGGAGAGGAGAGGTGGACAAAGTACTTGGTTGCTAAGAACCTAAGAACGTGTAT    960
GCTTTGCTGAATTAGTCTGATAAGTGAATGTTTATCTATCTTTGTGGAAAACAGATAATG   1020
GAGTTGGGGCAGGAAGCCTATGCGCCATCCTCCAAAGACAGACAGAATCACCTGAGGCGT   1080
TCAAAAGATATAACCAAATAAACAAGTCATCCACAATCAAAATACAACATTCAATACTTC   1140
CAGGTGTGTCAGACTTGGGACGCTGATAGGGACGCTGATATAATAGGGTAGAAAGAAGTAACACGAA  1200
GAAGTGGTGGAAATGTAAAATCCAAGTCATATGGCAGTGATCAATTATTAATCAATTAAT   1260
AATATTAATAAATTTCTTATATTTAAAAAAAAAAAAAAAA 1302
```


SIGNAL SEQUENCE

TRANSMEMBRANE

HYDRO-PHOBIC

HYDRO-PHILIC

N   N   N   N

3   0   -3

0   50   100   150   200

FIG. 5a

```
EPCR    MLTT..LLPILLLSGWAFCSQDASDGLQRHMLQISYFRDPYHVWYQGNASLGGHLTHVL
CCD41   MLTKFLLLLLLLPGCAFVTPMAPKAAYAPDLL....FPRPPSCEASGQRSLGKLLTHTL
CD1d    MGC...LLFLLLWALLQAWGSAEVPQRLFPLRCLQISSFANSSWTRTDGLAWLGELQTHSW
MCD1.2  MRY...LPCLLLWAFLQVWGQSEVQQKNYTFRCLQTSSFANISWSRTDSLILLGDLQTHRW

EPCR    EGPDTNTTIIQLQPLQEPESWARTQSGLQSYLLQFHGLVRLVHQER......TLAFPLTI
CCD41   EGPSQNVTILQLQPWQDPESWERTESGLQIYLTQFESLVKLVYRER....KENVFFPLTV
CD1d    SN..DSDTVRSLKPWSQGTFSDQQWETLQHIFRVYRSSFTRDVKEFAKML..RSYPYEL
MCD1.2  SN..DSATISFTKPWSQGKLSNQQWEKLQHMFQVYRVSFTRDIQELVKMMSPKEDYPIEI

EPCR    RCFLGCEL.P..PEGSRAHVFFEVAVNGSSFVSFRPERALWQADTQVTSGVWTFTLQQL
CCD41   SCSLGCEL.PEEEEEGSEPHVFFDVAVNGSAFVSFRPKTAVWVSGSQEPSKAANFTLKQL
CD1d    QVSAGCEVHP...GNASNNFFHVAFQGKDILSFQGTS...WEP.TQEAPLWMNLAIQVL
MCD1.2  QLSTGCEMYP...GNASESFFHVAFQGKYAVVRFRGTS...WQR.VLGAPSWLDLPIKVL
```

SUBSTITUTE SHEET (RULE 26)

FIG. 5b

```
EPCR    NAYNRTRYELREFLEDTCVQYV.........QKHISAENTKGSQTS........
CCD41   NAYNRTRYELQEFLQDTCVEFL.........ENHITTQNMKGSQTG
CD1d    NQDKWTRETVQWLLNGTCPQFVSGLLESGKSELKQVKPKAWLSRGPSPGPGRLLLVCHVSG
MCD1.2  NADQGLSATVQTLLNDTWPQFARGLLEAGKSDLEKQEKPVAWLSSVPSSAHGHLQLVCHVSG

EPCR    ................
CCD41   ................
CD1d    FYPKPVWVKWMRGEQEQQGTQPGDILPNADETWYLRATLDVVAGEAAGLSCRVKHSSLEG
MCD1.2  FYPKPVWVMWMRGDQEQQGTHRGDFLPNADETWYLQATLDVEAGEEAGLACRVKHSSLGG

EPCR    ........RSYTSLVLGVLVGGFIIAGVAVGIFLCTGGRRC
CCD41   ........RSYTSLVLGILMGCFIIAGVAVGIFMCTSGRGLLII
CD1d    QDIVLYWGGSYTSMGLIALAVLACLLFLLIVGFTS.RFKRQTSYQGVL
MCD1.2  QDIILYWDARQAPVGLIIVFIVLIMLVVGAVVYYI.WRRRSAYQDIR
```

```
 1 MLTTLLP..ILLLSGWAFCSQDASDGLQRLHMLQISYFRDPYHVWYQGNA 48
   || |-|| :|||-||-|  |||-||-|| |||||||-|-::||:-||||
 1 MLTKFLPLLLLLLPGCALCN...SDGSQSLHMLQISYFQDHHHVRHQGNA 47

49 SLGGHLTHVLEGPDTNTTIIQLQPLQEPESWARTQSGLQSYLLQFHGLVR 98
   |||  |||-||||-|-||:|||-|-|||-||-|||||| ||--||:
48 SLGKLLTHTLEGPSQNVTILQLQPWQDPESWERTESGLQIYLTQFESLVK 97

99 LVHQER..TLAFPLTIRCFLGCELP...PEGSRAHVFFEVAVNGSSFVSF 143
   ||-||  -:||||--| |||||  -|||:|||-||||||-||||
98 LVYRERKENVFFPLTVSCSLGCELPEEEEEGSEPHVFFDVAVNGSAFVSF 147

144 RPERALWQADTQVTSGVVTFTLQQLNAYNRTRYELREFLEDTCVQYVQKH 193
    ||-|-|:-|-| |  ...|I-|||||||||||||| |||-||||----|
148 RPKTAVWVSGSQEPSKAANFTLKQLNAYNRTRYELQEFLQDTCVEFLENH 197

194 ISAENTKGSQTSRSYTSLVLGVLVGGFIIAGVAVGIFLCTGGRRC 238
    |--:| |||||-||||||||-|-|- |||||||||||-||-|||
198 ITTQNMKGSQTGRSYTSLVLGILMGCFIIAGVAVGIFMCTSGRRC 242
```

FIG. 6

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C12N15/12   C07K14/705   A61K39/395   C12N15/11   A61K38/17
         C07K16/28   G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   C12N   C07K   A61K   G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 42, 21 October 1994 BALTIMORE, MD, USA, pages 26486-26491, K. FUKUDOME ET AL. 'Identification, cloning, and regulation of a novel endothelial cell protein C/activated protein C receptor.' see the whole document	1-3,7-11

	-/--	

[X] Further documents are listed in the continuation of box C.   [ ] Patent family members are listed in annex.

Date of the actual completion of the international search	Date of mailing of the international search report
2 January 1996	26.01.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL · 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Nooij, F

C.(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	CIRCULATION, vol. 90, no. 4 part 2, October 1994 NEW YORK, NY, USA, page I-N K. FUKUDOME ET AL. 'Identification, cloning, and regulation of a novel endothelial cell protein C/activated protein C receptor.' see the whole document	1-3,7-11
P,X	CIRCULATION, vol. 90, no. 4 part 2, October 1994 NEW YORK, NY, USA, page I133 K. FUKUDOME ET AL. 'Identification, cloning and regulation of a novel endothelial cell protein C/activated protein C receptor.' see abstract 0707	1-3,7-11
P,X	THROMBOSIS AND HAEMOSTASIS, vol. 72, no. 3, September 1994 STUTTGART, GERMANY, pages 465-474, N. BANGALORE ET AL. 'High affinity binding sites for activated protein C and protein C on cultured human umbilical vein endothelial cells.' see summary see page 472, right column, line 9 - line 50	1-3,7-11
P,X	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 10, 10 March 1995 BALTIMORE, MD, USA, pages 5571-5577, K. FUKUDOME ET AL. 'Molecular cloning and expression of murine and bovine endothelial cell protein C/activated protein C receptor (EPCR).' see abstract see figures 1-5	1-4,7-12

1

INTERNATIONAL SEARCH REPORT

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:   16-18
because they relate to subject matter not required to be searched by this Authority, namely:

> Remark: Although these claims are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   [ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

CORRECTED VERSION

PCT

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :		(11) International Publication Number:	WO 96/05303
C12N 15/12, C07K 14/705, A61K 39/395, C12N 15/11, A61K 38/17, C07K 16/28, G01N 33/68	A1	(43) International Publication Date:	22 February 1996 (22.02.96)

(21) International Application Number: PCT/US95/09636

(22) International Filing Date: 9 August 1995 (09.08.95)

(30) Priority Data:
289,699   12 August 1994 (12.08.94)   US

(71) Applicant: OKLAHOMA MEDICAL RESEARCH FOUNDA-TION [US/US]; 825 N.E. Thirteenth Street, Oklahoma City, OK 73104 (US).

(72) Inventors: FUKUDOME, Kenji; 13125 West Park Place, Oklahoma City, OK 73142 (US). ESMON, Charles, T.; 5800 North Stonewall, Oklahoma City, OK 73111 (US).

(74) Agent: PABST, Patrea, L.; Arnall Golden & Gregory, 2800 One Atlantic Center, 1201 West Peachtree Street, Atlanta, GA 30309-3450 (US).

(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
    With international search report.
    Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: CLONING AND REGULATION OF AN ENDOTHELIAL CELL PROTEIN C/ACTIVATED PROTEIN C RECEPTOR

(57) Abstract

Human protein C and activated protein C were shown to bind to endothelium specifically, selectively and saturably (Kd=30 nM, 7000 sites per cell) in a Ca^{2+} dependent fashion. Expression cloning revealed a 1.3 kb cDNA that coded for a novel type I transmembrane glycoprotein capable of binding protein C. This protein appears to be a member of the CD1/MHC superfamily. Like thrombomodulin, the receptor involved in protein C activation, the endothelial cell protein C receptor (EPCR) function and message are both down regulated by exposure of endothelium to TNF. Identification of EPCR as a member of the CD1/MHC superfamily provides insights into the role of protein C in regulating the inflammatory response, and determination of methods for pharmaceutical use in manipulating the inflammatory response.

CLONING AND REGULATION OF AN ENDOTHELIAL CELL PROTEIN C/ACTIVATED PROTEIN C RECEPTOR

Background of the Invention

The present invention is generally in the area of cloning, expression, and regulation of an endothelial cell protein C/activated protein C

5   receptor.

Protein C plays a major role in the regulation of blood coagulation. Patients deficient in protein C usually exhibit life threatening thrombotic-complications in infancy

10   (Seligsohn et al., (1984) N. Engl. J. Med. 310, 559-562; Esmon, (1992) Trends Cardiovasc. Med. 2, 214-220) that are corrected by protein C administration (Dreyfus et al., (1991) N. Engl. J. Med. 325, 1565-1568). In addition, activated

15   protein C (APC) can prevent the lethal effects of E. coli in baboon models of gram negative sepsis (Taylor et al., (1987) J. Clin. Invest. 79; U.S. Patent No. 5,009,889 to Taylor and Esmon) and preliminary clinical results suggest that protein C

20   is effective in treating certain forms of human septic shock (Gerson et al., (1993) Pediatrics 91, 418-422). These results suggest that protein C may both control coagulation and influence inflammation. Indeed, inhibition of protein S, an

25   important component of the protein C pathway, exacerbates the response of primates to sublethal levels of E. coli and augments the appearance of TNF in the circulation (Taylor et al., (1991) Blood 78, 357-363). The mechanisms involved in

30   controlling the inflammatory response remain unknown.

Protein C is activated when thrombin, the terminal enzyme of the coagulation system, binds to an endothelial cell surface protein, thrombomodulin

35   (Esmon, (1989) J. Biol. Chem. 264, 4743-4746; Dittman and Majerus, (1990) Blood 75, 329-336;

Dittman, (1991) _Trends Cardiovasc. Med._ _1_,
331-336). In cell culture, thrombomodulin
transcription is blocked by exposure of endothelial
cells to tumor necrosis factor (TNF) (Conway and

5   Rosenberg, (1988) _Mol. Cell. Biol._ _8_, 5588-5592)
and thrombomodulin activity and antigen are
subsequently internalized and degraded (Lentz et
al., (1991) _Blood_ 77, 543-550, Moore,K.L., et.al.,
(1989) _Blood_ 73, 159-165). In addition, C4bBP,

10   a regulatory protein of the complement system,
binds protein S to form a complex that is
functionally inactive in supporting APC
anticoagulant activity _in vitro_ (Dahlbäck, (1986)
J. Biol. Chem. _261_, 12022-12027) and _in vivo_

15   (Taylor,et al., 1991). Furthermore, C4bBP behaves
as an acute phase reactant (Dahlbäck, (1991)
Thromb. Haemostas. _66_, 49-61). Thus, proteins of
this pathway not only appear to regulate
inflammation, but they also interact with

20   components that regulate inflammation, and they
themselves are subject to down regulation by
inflammatory mediators.
          Given the central role of the protein C
pathway in regulating the host response to

25   inflammation and the critical role of the pathway
in controlling blood coagulation, it is important
to identify and characterize all of the components
that interact with the system. This is especially
true since the molecular basis of the

30   anti-inflammatory effects of the protein C pathway
components have yet to be elucidated at the
molecular level.
          It is therefore an object of the present
invention to provide a cellular receptor for

35   protein C and activated protein C.
          It is a further object of the present
invention to provide nucleotide sequences encoding

the cellular receptor and amino acid
characterization of the receptor which allows
expression of recombinant native and modified forms
of the receptor.

5   It is another object of the present
invention to provide methods of modulating the
inflammatory response involving protein C and
activated protein C.

Summary of the Invention

10   An endothelial cell protein C binding
protein (referred to herein as "EPCR") has been
cloned and characterized. The protein is predicted
to consist of 238 amino acids, which includes a 15
amino acid signal sequence at the N-terminus, and a
15   23 amino acid transmembrane region which
characterizes the receptor as a type 1
transmembrane protein. The protein binds with high
affinity to both protein C and activated protein C
(Kd=30 nM) and is calcium dependent. The message
20   and binding function of the receptor are both down
regulated by cytokines such as TNF.

These results identify a new member of a
complex pathway that, like other members of the
pathway, is subject to regulation by inflammatory
25   cytokines, and can therefore be used to modulate
inflammatory reactions in which protein C or
activated protein C is involved. Inhibition of
the inflammatory response can be obtained by
infusing soluble EPCR. Alternatively, localizing
30   EPCR to surfaces in contact with blood will render
the surfaces anticoagulant by virtue of the ability
of EPCR to bind and concentrate the anticoagulant
activated protein C at the surface. Alternatively,
the function of EPCR can be enhanced by
35   overexpressing the EPCR in endothelium that could

4

be used to coat vascular grafts in patients with vascular disease or on stents in cardiac patients.

Brief Description of the Drawings

5   Figures 1A, 1B and 1C are flow cytometric analyses of Fl-APC (fluorescent labelled activated protein C) binding to HUVEC (human umbilical vein endothelial cells). Figure 1A is a graph of cell number versus log of fluorescence intensity, demonstrating Fl-APC binding to HUVEC. HUVEC (1 x

10   10^5) were incubated at room temperature without (dotted line) or with 160 nM of Fl-APC (solid line) in the presence of 1.3 mM $CaCl_2$. After washing, bound APC was analyzed by flow cytometry. Figure 1B is a graph of fluorescence intensity versus Fl-

15   APC concentration (nM) demonstrating the concentration dependence of Fl-APC binding to HUVEC. HUVEC were incubated with Fl-APC in the absence (open circles) or presence of 1.3 mM $CaCl_2$ (closed circles) and binding was measured as in A.

20   Mean channel fluorescence intensity is plotted for each Fl-APC concentration (between 0 and 800 nM). Figure 1C is a graph of the percent of mean fluorescence versus unlabeled protein concentration ($\mu g/ml$), demonstrating the effects of unlabeled

25   proteins on Fl-APC binding to HUVEC. Fl-APC binding to HUVEC was carried out in the presence of the indicated concentrations (between 0 and 100 $\mu g/ml$) of unlabeled APC, protein C, protein S, factor X and Xa or recombinant Gla-domainless

30   protein C (rGDPC).

Figures 2A, 2B, 2C and 2D are graphs of ^{125}I-APC Binding to HUVEC Monolayers. Figure 2A is a graph of the bound APC (cpm x 10^{-3}) versus time (min), showing the time course of ^{125}I-APC binding

35   to HUVEC. HUVEC monolayers (1.2 x 10^5 cells) were incubated at 4°C with 32 nM (filled squares) or 8

5

nM (op n squares) ^{125}I-APC. At the indicated times,
cells were washed and bound radioactivity was
measured. Figure 2B is a graph of bound APC (cpm x
10^{-3}) versus unlabeled protein (nM) demonstrating

5   the effects of unlabeled APC and rGDPC on ^{125}I-APC
binding to HUVEC. HUVEC were incubated at 4°C for
one hour with ^{125}I-labeled APC in the presence of
the indicated concentrations (between 01 and
approximately 1000 nM) of unlabeled APC (open

10   circles) or rGDPC (closed circles). After washing,
bound radioactivity was measured. Figure 2C is a
graph of bound APC (fmol/well) versus free APC (nM)
demonstrating the concentration dependence of ^{125}I-
APC binding to HUVEC. Monolayers of HUVEC were

15   incubated with the concentrations of ^{125}I-APC
indicated as described above. Specific binding was
determined as described below. Figure 2D is a
Scatchard analysis of ^{125}I-APC binding to HUVEC.
Each value was calculated from the data shown in

20   Figure 2C.

Figures 3A and 3B are flow cytometric
analyses of F1-APC binding to 293T cells
transfected with a cDNA clone of EPCR. Cells were
transfected with a clone EPCR/pEF-BOS or pEF-BOS

25   (negative control) by the calcium/phosphate method.
After 24 h, cells were harvested and F1-APC binding
was performed in the absence (dotted lines) or
presence of 1.3 mM CaCl$_2$ (solid lines).

Figures 4a-4c are the predicted protein
30   structure of EPCR based on nucleotide sequence (SEQ
ID NO. 1), predicted amino acid sequence (SEQ ID
NO. 2) and a hydropathy plot of EPCR. The signal
sequence and transmembrane region are indicated
with the solid bars.

35   Figures 5a-5b are a comparison of the amino
acid sequence of EPCR to the amino acid sequences
of other members of the CD1 family and CCD41. The
EPCR sequence is shown in the first line and

SUBSTITUTE SHEET (RULE 26)

compared to murine CCD41 (second line), human CD1d (third line) and murine CD1.2 (fourth line). Identities with EPCR are indicated by open boxes. Residues that are conserved between EPCR and all of

5 the human CD1 family members are indicated by a double asterisk. Residues shared with one or more members of the CD1 family are indicated by a single asterisk.

Figure 6 is a comparison of the amino

10 acid sequence of human EPCR (first line) to the amino acid sequence of murine EPCR (second line). Identities are indicated by lines. Similarities are indicated with dots.

Detailed Description of the Invention

15 I.   Cloning and Characterization of EPCR.

Human protein C and activated protein C are shown to bind to endothelium specifically, selectively and saturably (Kd = 30 nM, 7000 sites per cell) in a Ca^{2+} dependent fashion. FL-APC

20 binding to various human cell lines were examined, and found that the binding was HUVEC specific. A human kidney cell line transformed with SV40 large T antigen, 293T cells, expressed very few of these binding sites. A HUVEC cDNA library was

25 constructed using the powerful mammalian expression vector, pEF-BOS (Mizushima and Nagata, (1990) Nucleic Acids Res. 18, 5322). Plasmid DNA was prepared from subpools of independent colonies (2,500 colonies per pool), and transfected into

30 293T cells, using the method of Kaisho et al., (1994) Proc. Natl. Acad. Sci. (USA) 91, 5325. FL-APC binding was analyzed on a flow cytometer. One of eight subpools gave a positive signal. This subpool was divided into 20 subpools and

35 rescreened. After three rounds of enrichment, one positive clone, EPCR-1, was isolated. EPCR-1

RECTIFIED SHEET (RULE 91)
ISA/EP

7

carries a 1.3 kb insert. When transfected into
293T cells. this clone was capable of expressing
the calcium-dependent binding site for FL-APC on
the 293T cell surface.

5   Expression cloning revealed a 1.3 kb cDNA
that coded for a type I transmembrane glycoprotein
capable of binding protein C. This protein appears
to be a member of the CD1/MHC superfamily. Like
thrombomodulin, the receptor involved in protein C
10 activation, the endothelial cell protein C receptor
(EPCR) function and message are both down regulated
by exposure of endothelium to TNF. Identification
of EPCR as a member of the CD1/MHC superfamily
provides insights into the role of this receptor
15 for protein C in regulating the inflammatory
response.

                    Materials and Methods
                    Protein Preparation
            Human protein C (Esmon et al., (1993)
20 Meths. Enzymol. 222, 359-385), APC (Esmon et al.,
1993), recombinant gla domainless protein C (rGDPC)
(Rezaie et al., (1992) J. Biol. Chem. 267,
11701-11704), protein S (Taylor et al., 1991),
factor X and factor Xa (Le Bonniec et al., (1992)
25 J. Biol. Chem. 267, 6970-6976) were prepared as
described in the cited publications.

            Selective labeling of the active site of
APC with fluorescein was performed by the method of
Bock (Bock, P.E. (1988) Biochemistry 27,
30 6633-6639). In brief, N^{α}-
[(acetylthio)acetyl]-D-Phe-Pro-Arg-CH_2Cl, (200 μM)
was reacted with 40 μM APC for 1 hour at room
temperature. After dialysis, the covalently
modified APC was incubated at room temperature for
35 one hour with 200 μM 5-(iodoacetamido)fluorescein
(Molecular Probes). Free fluorescein was removed
by gel filtration on a PD-10 column (Pharmacia).

With this method, each molecule of fluoresceinated
APC (Fl-APC) contains a single dye at the active
site and hence all of the fluorescent molecules
behave identically.

5   Iodogen (Pierce) was used to radiolabel
APC with Na[^{125}I] (Amersham) according to the
manufacture's protocol in the presence of 5 mM
CaCl$_2$. Free ^{125}I was removed by gel filtration on a
PD-10 column. The specific activity of the ^{125}I-APC

10   was 1 x 10^4 cpm/ng protein.

Cell Culture

Human umbilical vein endothelial cells
(HUVEC) were isolated from fresh umbilical-cords by
collagenase treatment and cultured in medium 199

15   containing 15% fetal bovine serum, 10 μg/ml
heparin, and 0.5% endothelial cell growth
supplement prepared from bovine brain extract
(Maciag at al., (1979) Proc. Natl. Acad. Sci. (USA)
76, 5674-5678). HOS (ATCC CRL 1543), HEp-2 (ATCC

20   CCL 23) and 293 cells (ATCC CRL 1573) transformed
with SV40 large T antigen (293T, a gift from Dr.
Kenji Oritani) were maintained in Earl's MEM
supplemented with 10% fetal bovine serum. The
human lymphocyte cell lines, Jurkat, MOLT3 (ATCC

25   CRL 1552), Jijoye (ATCC CCL 87), Raji (ATCC CCL
86), U-937 (ATCC CRL 1593), HL-60 (ATCC CCL 240),
and HEL (ATCC TIB 180), were maintained in
RPMI-1640 medium supplemented with 10% fetal bovine
serum.

30   ## Flow Cytometric Analysis of Fl-APC
Binding to Cells

Adherent cells were harvested by
incubation at 37°C for 5 min in phosphate buffered
saline (PBS) containing 0.02% EDTA. Cells were

35   washed twice with EDTA/PBS and then once with
Hank's balanced salt solution (HBSS). They were
resuspended in HBSS containing 1% bovine serum
albumin (BSA) and 0.02% sodium azide (binding

buffer). Cells (1 x 10⁵) were incubated at room
temperature for 45 min with Fl-APC in the dark.
After washing, they were resuspended in the binding
buffer containing 0.5 µg/ml of propidium iodide.

5   Bound Fl-APC was analyzed on a flow cytometer,
FACScan (Becton Dickinson). Living cells were
gated on a dot plot display of forward-scatter
(FSC) versus fluorescence-2 (FL2), and F1-APC
binding was detected on the fluorescence-1 (FL-1)

10   channel. All experiments were performed in
duplicate.

¹²⁵I-APC binding to HUVEC

Monolayers of HUVEC in 24-well
microplates (Costar) (1 x 10⁵ cells per well) were

15   washed twice with EDTA/PBS and once with ice-cold
HBSS. Cells were then incubated at 4°C for one
hour in the binding buffer with ¹²⁵I-APC. After
washing three times with ice-cold HBSS, cells were
released with the EDTA buffer, and the bound

20   radioactivity was measured in a gamma counter
(Isodata 500). To determine non-specific,
calcium-independent adsorption of radioactivity,
the cells were washed with EDTA/PBS and residual
radioactivity in the cell pellet was measured.

25   Non-specific binding of radioactivity was
consistently less than 5% of the specific binding.
The data was analyzed using the Enzfitter program
(Elsevier Biosoft, Cambridge, U.K.).

Construction of HUVEC cDNA Library

30   Poly-A RNA was isolated from HUVEC (1 x
10⁸ cells) using the FastTrack™ mRNA isolation kit
(Invitrogen). cDNA was synthesized from 3 µg of
poly-A RNA using a Librarian™ I kit (Invitrogen).
A BstX I adaptor was ligated, double stranded cDNA

35   was fractionated by agarose gel electrophoresis,
and cDNA longer than 700 bp was ligated into a
mammalian expression vector, pEF-BOS (Mizushima and

10

Nagata, 1990; this vector was a kind gift from Dr.
S. Nagata). The construct was transfected into *E.
coli* DH10B by electroporation (Bio-Rad Gene
Pulser™). The library consisted of 8 X 10⁶

5   independent colonies with an average size of 2.0
kb.

Expression Cloning and Sequence Analysis

Approximately 2 x 10⁴ independent colonies
were divided into eight subpools (each containing

10   2,500 independent colonies) and plasmid DNA was
prepared from each subpool. Sub-confluent 293T
cells in 24-well microplates were transfected with
1 μg of the DNA by the calcium/phosphate method
(Graham and Van Der Eb, (1973) Virology 52, 456-

15   467). After 20 hours, the medium was changed, and
culture was continued for another 24 hours. The
subpools were screened for F1-APC binding by FACS
analysis as described above. The positive library
pool was then divided into 20 new pools and

20   rescreened. After three rounds of screening, 96
individual clones were tested and one positive
clone was identified.

The insert (1.3 kb) was subcloned into
pBluescript™ (Stratagene), and the nucleotide

25   sequence was determined using a Sequenase™ version
2.0 DNA Sequencing kit (USB). Nucleotide and
protein database search employed the BLAST™ (NCBI)
and FASTA™ programs (GCG) with GenBank, EMBL, and
SwissProt databases.

30   ### Northern Blot Analysis

Total RNAs (15 μg) from various cells
were isolated, electrophoresed through formaldehyde
agarose gels and transferred to a nylon membrane
(Hybond-N™, Amersham). The 483 bp *Xba* I fragment

35   from the 5' end of the EPCR cDNA was labeled by
random priming according to the manufacturer's

11

instructions (Multiprime™ DNA labeling system,
Amersham) and used for hybridization.

Protein C and APC Binding to HUVEC

Endothelial cells in suspension bound
5   FL-APC, as monitored by flow cytometry, and
demonstrated in Figure 1A. Binding was saturable
and Ca^{2+} dependent, as shown by Figure 1B. Optimal
binding required at least 1 mM Ca^{2+}. FL-APC was
displaced from the cell surface by APC and protein
10   C equivalently, as shown by Figure 1C. The
homologous Gla-domain containing proteins, protein
S, factor X, and its active form, factor Xa, failed
to displace bound Fl-APC, suggesting that there is
a specific binding site for APC on the endothelial
15   cell surface. Protein C binding was dependent on
the Gla domain, since recombinant gla-domainless
protein C (rGDPC) failed to displace Fl-APC.

Detailed binding studies were also
performed with ^{125}I-labeled APC and monolayers of
20   HUVEC, as shown by Figures 2A, 2B, 2C and 2D. The
binding analysis indicated 7,000 sites per cell and
a Kd=30 nM. This affinity is similar to that
estimated from Figure 1.

Endothelial cell surface thrombomodulin
25   can interact with protein C and APC. The Kd
(greater than 1 μM) (Hogg et al., (1992) <u>J. Biol.</u>
<u>Chem.</u> 267, 703-706; Olsen et al., (1992)
<u>Biochemistry</u> 31, 746-754), however, is much higher
than that of the binding site described above with
30   respect to the new receptor. Furthermore,
polyclonal and monoclonal antibodies against
thrombomodulin that inhibit protein C activation
did not inhibit the binding. Protein S also can
interact with protein C and APC (Dahlbäck et al.,
35   (1992) <u>Biochemistry</u> 31, 12769-12777), but Fl-APC
binding to HUVEC was not influenced by protein S
addition. Furthermore, polyclonal and monoclonal

antibodies to protein S did not inhibit th
binding.   These results indicate the binding site
for protein C and APC on endothelium is distinct
from these known molecules.

5   **Nucleotide and Predicted Protein
        Structure Analysis of EPCR**

        The insert was subcloned into
pBluescript, and the nucleotide sequence was
determined, as shown in Sequence ID No. 1.   The

10   cDNA shown in Sequence ID No. 1 consists of 1302
bp, including a translation initiation ATG codon
(AGGATGT, (Kozak, (1986) _Cell_ 44, 283-292) at the
5'-end at nucleotides 25-27 of Sequence ID No. 1.
A potential polyadenylation signal sequence,

15   AATAAA, (Proudfoot and Brownlee, (1976) _Nature_ 263,
211-214) begins at nucleotide 1267 of Sequence ID
No. 1, just 18 bp upstream of the poly(A) sequence.
        The cDNA is predicted to code for a
protein of 238 amino acids (Sequence ID No. 2),

20   which includes a 15 amino acid signal sequence (von
Heijne, (1986) _Nucleic Acids Res._ 14, 4683-4690) at
the N-terminal.   Therefore, the mature protein is
predicted to contain 223 amino acids.   Sequence ID
No. 2 is the predicted amino acid sequence of EPCR.

25   Amino acids 1-15 of Sequence ID No. 2
(MLTTLLPILLLSGWA) are the putative signal sequence
determined by the method of von Heijne (von Heijne,
1986).   Amino acids 211-236 of Sequence ID No. 2
(LVLGVLVGGFIIAGVAVGIFLCTGGR) are the putative

30   transmembrane domain.   Potential N-glycosylation
sites are present at amino acids 47-49, 64-66, 136-
138, and 172-174 of Sequence ID No. 2.
Extracellular cysteine residues are present at
amino acids 17, 114, 118, and 186 of Sequence ID

35   No. 2.   A potential transmembrane region (Engelman
et al., (1986) _Annu. Rev. Biophys. Chem._ 15, 321-
53) consisting of 23 amino acids was identified at

the C-terminal end (beginning at amino acid 216 of
Sequence ID No. 2).

    The protein is predicted to be a type 1
transmembrane protein. The extracellular domain
5 contains four potential N-glycosylation sites and
four Cys residues. The cytoplasmic region contains
only three amino acids and terminates with a Cys,
which could be acylated to something or involved in
heterodimer formation with another peptide.

10   Although described with reference to
cloning and expression of the protein encoding
sequence, larger amounts of protein can be obtained
by expression in suitable recombinant host systems,
such as mammalian, yeast, bacteria, or insect

15 cells. Isolation can be facilitated by making
antibodies to the recombinant protein which are
then immobilized on substrates for use in
purification of additional receptors, as described
below.

20   As used herein, the nucleotide sequences
encoding the receptor include the sequence shown in
Sequence ID No. 1, and sequences having
conservative substitutions, additions or deletions
thereof which hybridize to Sequence ID No. 1 under

25 stringent conditions. As used herein, the amino
acids sequences constituting the receptor include
the sequence shown in Sequence ID No. 2, and
sequences having conservative substitutions,
additions or deletions thereof which form a

30 receptor having functionally equivalent biological
activity. It is well known to those skilled in the
art what constitutes conservative substitutions,
additions or deletions, and which could be readily
ascertained as encoding, or forming, a functionally

35 equivalent receptor molecule using the functional
assays described herein.

14

The hydropathic plot shown in Figures 4a-
4c was performed according to the method of Goldman
et al (Engelman et al., 1994) (solid line) and that
of Kyte and Doolittle (1982) J. Mol. Biol. 157,
5   105-132 (dotted line).

DNA and protein database searches
revealed that the sequence is related to the
centrosome-associated, cell cycle dependent murin
protein, CCD41, also referred as centrocyclin
10   (Rothbarth et al., (1993) J. Cell Sci. 104, 19-30),
as shown by Figures 5a-5b. The similarity in the
published sequence of murine CCD41 with human EPCR
led to the cloning and sequencing of the murine
EPCR. The sequence of murine EPCR is shown in
15   Figure 6. It is distinct from the published
sequence of CCD41.

The EPCR amino acid sequence was also
related to, but quite distinct from, the CD1/MHC
superfamily and the murine CD1.2, as also shown by
20   Figures 5a-5b. Based on the homology to CD1/MHC,
it is likely that EPCR contains two domains
consisting of residues 17-114 and 118-188. Of the
CD1 family members, CD1d is the most similar to
EPCR. In the mouse, CCD41 is associated
25   exclusively with the centrosome during G_1 but
becomes detectable elsewhere during the cell cycle,
reaching a maximum during G_2, except during the G_2/M
phase (Rothbarth et al., 1993). EPCR expression
appears restricted to endothelium, which would not
30   be expected for a cell cycle associated protein.

The identification of the protein C
receptor on endothelium suggests that the
endothelial cell binds protein C/APC through three
distinct mechanisms. In addition to EPCR, protein
35   S can bind APC/protein C on negatively charged
membrane surfaces that include the endothelium
(Stern et al., (1986) J. Biol. Chem. 261, 713-718),

RECTIFIED SHEET (RULE 91)
ISA/EP

but this is not cell-type specific (Dahlbäck et
al., 1992). Thrombomodulin in complex with
thrombin can bind protein C and APC (Hogg et al.,
1992). On endothelium, the protein S binding sites
(Nawroth and Stern, (1986) J. Exp. Med. 163,
740-745), thrombomodulin (Esmon, 1989) and EPCR are
all down regulated by cytokines, indicating that
inflammation can impair protein C pathway function
at multiple levels.

The homology to the CD1/MHC family of
proteins is especially interesting since it
provides indications as to the function of EPCR.
The CD1/MHC family has three extracellular domains
termed α1,2 and 3. The extracellular domain of EPCR
contains four Cys residues that appear to
correspond to two distinct domains. EPCR lacks the
third domain of the CD1/MHC family, but the two
domains have significant homology to the α1 and α2
domains of the CD1 protein family and the α2 domain
of the MHC class 1 protein, suggesting that these
proteins evolved from a common ancestor. The first
domain of EPCR, residues 17-114, contains two
potential N glycosylation sites and is rich in ß
strand structure, suggesting that it may form a ß
sheet. Despite the ß strand structure, consensus
sequences (Williams and Barclay, (1988) Ann. Rev.
Immunol. 6, 381-405) for the immunoglobulin
superfamily of receptors are absent. The second
domain of EPCR, residues 118-188, contains two
additional N glycosylation sites and, like the
CD1/MHC family, this domain is predicted to have
limited ß structure.

II.   Modulation of Inflammation using EPCR.

In vitro studies have suggested
anti-inflammatory activities for APC. For
instance, an unusual carbohydrate sequence on
protein C can inhibit inflammatory cell adhesion to

16

selectins (Grinnell at al., (1994) Glycobiology, 4,
221-226). Modest inhibitory effects of APC have
been reported on TNF production (Grey et al.,
(1993) Transplant. Proc. 25, 2913-2914). EPCR
5   could contribute to these anti-inflammatory
mechanisms. Since the homologous protein family,
CD1, can be linked to CD8 (Ledbetter et al., (1985)
J. Immunol. 134, 4250-4254), it is also possible
that the proteins C receptor is linked to another
10   protein and signal through this second protein.
One of the CD1 family members, CD1d, has been
reported to promote T cell responses, possibly
involving binding to CD8 (Panja et al., (1993) J.
Exp. Med. 178, 1115-1119). CD1b has recently been
15   reported to serve as an antigen presenting molecule
(Porcelli et al., (1992) Nature 360, 593-597). The
ability to bind protein C/APC could then be linked
either directly or indirectly to signalling via
direct interaction with cells of the immune system.
20   Since the MHC class of proteins is involved in
presentation of proteins to cell receptors, the
concept of presentation of protein C/APC to
inflammatory cells as a means of elaborating
anti-inflammatory activity may also be involved.
25   This includes modulation of enzyme specificity such
as occurs with thrombin-thrombomodulin interaction
(Esmon, 1989). In this case, the EPCR-APC complex
might cleave biologically active peptides from
unknown substrates.
30   **EPCR mRNA Levels and APC Binding**
To determine the cellular specificity of
EPCR expression, the intensity of FL-APC binding to
HUVEC was compared to several human cell lines.
Fl-APC bound strongly only to HUVEC, and not to any
35   of the T, B, or monocytic cell lines tested. Cells
were incubated at room temperature without or with
160 nM Fl-APC in the presence of 1.3 mM $CaCl_2$.

17

Binding was analyzed by flow cytometry. Slight
binding was demonstrated with the osteosarcoma
line, HOS and the epidermoid carcinoma cell line,
HEp-2.

5   Total RNA was extracted from these cells
and hybridized with the EPCR cDNA probe for
Northern Blot Analysis. EPCR mRNA was detected by
Northern blot analysis for HUVEC, Jurkat, HEp-2,
Raji, HOS, and U937. Among the cells lines tested,

10 EPCR mRNA was detected at high levels only in
HUVEC. The calculated mRNA size of 1.3 kb was
identical to the size of the isolated cDNA. After
prolonged exposure, a weak signal was also detected
with the osteosarcoma cell line HOS and monocyte

15 cell line U937. Thus, both APC binding and EPCR
mRNA expression are very specific for endothelium.

**Effects of TNF on APC Binding and EPCR
MRNA Levels**

Several other members of the protein C

20 anticoagulant pathway are subject to regulation by
inflammatory cytokines (Esmon, 1989). For
instance, endothelial cell surface thrombomodulin
expression and message are known to be reduced by
exposure of the cells to TNF (Conway and Rosenberg,

25 1988; Lentz et al., 1991). To determine if a
similar process occurs with EPCR, HUVEC were
treated with TNF and APC binding and expression of
EPCR mRNA were examined. APC binding to HUVEC
decreased in a time dependent fashion. EPCR

30 activity decreased more rapidly than thrombomodulin
antigen. HUVEC were cultured for 0, 6, 24 and 48
hr, in the presence of TNF-α (10 ng/ml). Cells
were harvested and residual F1-APC binding or
thrombomodulin (TM) expression was analyzed by flow

35 cytometry. Cell surface TM was stained with an
anti-TM murine monoclonal antibody and FITC--
conjugated anti-mouse IgG. The negative control is
without added fluorescent ligand.

18

HUVEC were treated with 10 ng/ml of TNF-α
for 0, 0.5, 1, 2, 3, 6, 10 and 24 hr, and message
was extracted and detected as described above. The
results demonstrated that the concentration of EPCR
5   mRNA was also reduced by TNF treatment. Message
levels and APC binding activity decreased in
parallel. Therefore, the TNF mediated
down-regulation of APC binding to endothelium
probably occurs at the level of mRNA expression.
10   Enhancement of inflammatory responses by
blocking binding of endogenous molecules to ECPCR
can be achieved by administration of compounds
binding to the receptor to a subject in need of
inhibition. The degree of binding is routinely
15   determined using assays such as those described
above. Compounds which are effective include
antibodies to the protein, fragments of antibodies
retaining the binding regions, and peptide
fragments of APC which include the Gla region.
20   Inhibition of the inflammatory response could be
obtained by infusing soluble EPCR. Alternatively,
localizing EPCR to surfaces in contact with blood
would render the surfaces anticoagulant by virtue
of the ability of EPCR to bind and concentrate the
25   anticoagulant APC at the surface. Alternatively,
the function of EPCR could be enhanced by
overexpressing the EPCR in endothelium used to coat
vascular grafts in patients with vascular disease
or on stents in cardiac patients.
30   The DNA sequence can also be used for
screening for other homologous or structurally
similar receptor proteins using hybridization
probes.
          These methods and reagents and
35   pharmaceuticals are more readily understood by
reference to the following.

Screening of patient samples for expression of receptor proteins.

Patients with thrombosis or hyperinflammatory conditions could be screened for defects in the EPCR gene. Sequence ID No. 1, and consecutive portions thereof of at least about seven nucleotides, more preferably fourteen to seventeen nucleotides, most preferably about twenty nucleotides, are useful in this screening using hybridization assays of patient samples, including blood and tissues. Screening can also be accomplished using antibodies, typically labelled with a fluorescent, radiolabelled, or enzymatic label, or by isolation of target cells and screening for binding activity, as described in the examples above. Typically, one would screen for expression on either a qualitative or quantitative basis, and for expression of functional receptor. Labelling can be with ^{32}P, ^{35}S, fluorescein, biotin, or other labels routinely used with methods known to those skilled in the art for labelling of proteins and/or nucleic acid sequences.

Hybridization Probes

Reaction conditions for hybridization of an oligonucleotide probe or primer to a nucleic acid sequence vary from oligonucleotide to oligonucleotide, depending on factors such as oligonucleotide length, the number of G and C nucleotides, and the composition of the buffer utilized in the hybridization reaction. Moderately stringent hybridization conditions are generally understood by those skilled in the art as conditions approximately 25°C below the melting temperature of a perfectly base-paired double-stranded DNA. Higher specificity is generally achieved by employing incubation conditions having higher temperatures, in other words, more stringent conditions. In general, the longer the sequence

or higher the G and C content, the higher the
temperature and/or salt concentration required.
Chapter 11 of the well-known laboratory manual of
Sambrook et al., MOLECULAR CLONING: A LABORATORY MANUAL,
5   second edition, Cold Spring Harbor Laboratory
Press, New York (1990), (which is incorporated by
reference herein), describes hybridization
conditions for oligonucleotide probes and primers
in great detail, including a description of the
10   factors involved and the level of stringency
necessary to guarantee hybridization with
specificity.

The preferred size of a hybridization
probe is from 10 nucleotides to 100,000 nucleotides
15   in length. Below 10 nucleotides, hybridized
systems are not stable and will begin to denature
above 20°C. Above 100,000 nucleotides, one finds
that hybridization (renaturation) becomes a much
slower and incomplete process, as described in
20   greater detail in the text MOLECULAR GENETICS, Stent,
G.S. and R. Calender, pp. 213-219 (1971). Ideally,
the probe should be from 20 to 10,000 nucleotides.
Smaller nucleotide sequences (20-100) lend
themselves to production by automated organic
25   synthetic techniques. Sequences from 100-10,000
nucleotides can be obtained from appropriate
restriction endonuclease treatments. The labeling
of the smaller probes with the relatively bulky
chemiluminescent moieties may in some cases
30   interfere with the hybridization process.

Generation of Antibodies for
Diagnostic or Therapeutic Use
Antibodies to the receptor proteins can
also be generated which are useful in detection,
35   characterization or isolation of receptor proteins,
as well as for modifying receptor protein activity,
in most cases, through inhibition of binding.
Antibodies are generated by standard techniques,

using human or animal receptor proteins. Since the
proteins exhibit high evolutionary conservation, it
may be advantageous to generate antibodies to a
protein of a different species of origin than the
5   species in which the antibodies are to be tested or
utilized, looking for those antibodies which are
immunoreactive with the most evolutionarily
conserved regions. Antibodies are typically
generated by immunization of an animal using an
10   adjuvant such as Freund's adjuvant in combination
with an immunogenic amount of the protein
administered over a period of weeks in two to three
week intervals, then isolated from the serum, or
used to make hybridomas which express the
15   antibodies in culture. Because the methods for
immunizing animals yield antibody which is not of
human origin, the antibodies could elicit an
adverse effect if administered to humans. Methods
for "humanizing" antibodies, or generating less
20   immunogenic fragments of non-human antibodies, are
well known. A humanized antibody is one in which
only the antigen-recognized sites, or
complementarily-determining hypervariable regions
(CDRs) are of non-human origin, whereas all
25   framework regions (FR) of variable domains are
products of human genes. These "humanized"
antibodies present a lesser xenographic rejection
stimulus when introduced to a human recipient.
           To accomplish humanization of a selected
30   mouse monoclonal antibody, the CDR grafting method
described by Daugherty, et al., (1991) *Nucl. Acids
Res.*, 19:2471-2476, incorporated herein by
reference, may be used. Briefly, the variable
region DNA of a selected animal recombinant anti-
35   idiotypic ScFv is sequenced by the method of
Clackson, T., et al., (1991) *Nature*, 352:624-688,
incorporated herein by reference. Using this

22

sequence, animal CDRs are distinguished from animal
framework regions (FR) based on locations of the
CDRs in known sequences of animal variable genes.
Kabat, H.A., et al., Sequences of Proteins of
5　Immunological Interest, 4th Ed. (U.S. Dept. Health
and Human Services, Bethesda, MD, 1987). Once the
animal CDRs and FR are identified, the CDRs are
grafted onto human heavy chain variable region
framework by the use of synthetic oligonucleotides
10　and polymerase chain reaction (PCR) recombination.
Codons for the animal heavy chain CDRs, as well as
the available human heavy chain variable region
framework, are built in four (each 100 bases long)
oligonucleotides. Using PCR, a grafted DNA
15　sequence of 400 bases is formed that encodes for
the recombinant animal CDR/human heavy chain FR
protection.

　　　　　The immunogenic stimulus presented by the
monoclonal antibodies so produced may be further
20　decreased by the use of Pharmacia's (Pharmacia LKB
Biotechnology, Sweden) "Recombinant Phage Antibody
System" (RPAS), which generates a single-chain Fv
fragment (ScFv) which incorporates the complete
antigen-binding domain of the antibody. In the
25　RPAS, antibody variable heavy and light chain genes
are separately amplified from the hybridoma mRNA
and cloned into an expression vector. The heavy
and light chain domains are co-expressed on the
same polypeptide chain after joining with a short
30　linker DNA which codes for a flexible peptide.
This assembly generates a single-chain Fv fragment
(ScFv) which incorporates the complete antigen-
binding domain of the antibody. Compared to the
intact monoclonal antibody, the recombinant ScFv
35　includes a considerably lower number of epitopes,
and thereby presents a much weaker immunogenic
stimulus when injected into humans.

The antibodies can be formulated in standard pharmaceutical carriers for administration to patients in need thereof. These include saline, phosphate buffered saline, and other aqueous carriers, and liposomes, polymeric microspheres and other controlled release delivery devices, as are well known in the art. The antibodies can also be administered with adjuvant, such as muramyl dipeptide or other materials approved for use in humans (Freund's adjuvant can be used for administration of antibody to animals).

Screening for drugs modifying or altering the extent of receptor function or expression

The receptor proteins are useful as targets for compounds which turn on, or off, or otherwise regulate binding to these receptors. The assays described above clearly provide routine methodology by which a compound can be tested for an inhibitory effect on binding of PC or APC. The in vitro studies of compounds which appear to inhibit binding selectively to the receptors are then confirmed by animal testing. Since the molecules are so highly evolutionarily conserved, it is possible to conduct studies in laboratory animals such as mice to predict the effects in humans.

In cases where inflammatory mediators or vascular disease down regulate EPCR, it would be advantageous to increase its concentration in vivo on endothelium. The binding assays described here and the gene sequence allow assays for increased EPCR expression. Similar approaches have been taken with thrombomodulin and investigators have shown that cyclic AMP (Maruyama, I. et al. (1991) Thrombosis Research 61, 301-310) and interleukin 4 (Kapiotis, S. et al., (1991) Blood 78, 410-415) can elevate thrombomodulin expression. The ability to

screen such drugs or drugs that block TNF down
regulation of EPCR provide an approach to elevating
EPCR expression in vivo and thus enhancing
anticoagulant and anti-inflammatory activity.

5   Studies based on inhibition of binding
are predictive for indirect effects of alteration
of receptor binding. For example, inhibition of
binding of APC or increased expression of TNF is
predictive of inhibition of EPCR function.

10   Assays for testing compounds for useful
activity can be based solely on interaction with
the receptor protein, preferably expressed on the
surface of transfected cells such as those
described above. Proteins in solution or
15 immobilized on inert substrates can also be
utilized. These can be used to detect inhibition
or enhancement in binding of PC or APC

Alternatively, the assays can be based on
interaction with the gene sequence encoding the
20 receptor protein, preferably the regulatory
sequences directing expression of the receptor
protein. For example, antisense which binds to the
regulatory sequences, and/or to the protein
encoding sequences can be synthesized using
25 standard oligonucleotide synthetic chemistry. The
antisense can be stabilized for pharmaceutical use
using standard methodology (encapsulation in a
liposome or microsphere; introduction of modified
nucleotides that are resistant to degradation or
30 groups which increase resistance to endonucleases,
such as phosphorothiodates and methylation), then
screened initially for alteration of receptor
activity in transfected or naturally occurring
cells which express the receptor, then in vivo in
35 laboratory animals. Typically, the antisense would
inhibit expression. However, sequences which block

25

those sequences which "turn off" synthesis can also
be targeted.

The receptor protein for study can be
isolated from either naturally occurring cells or
5   cells which have been genetically engineered to
express the receptor, as described in the examples
above. In the preferred embodiment, the cells
would have been engineered using the intact gene.

Random generation of receptor or
10   *receptor encoding sequence binding*
molecules.

Molecules with a given function,
catalytic or ligand-binding, can be selected for
from a complex mixture of random molecules in what
15 has been referred to as "in vitro genetics"
(Szostak, (1992) TIBS 19:89). One synthesizes a
large pool of molecules bearing random and defined
sequences and subjects that complex mixture, for
example, approximately 10^{15} individual sequences in
20 100 μg of a 100 nucleotide RNA, to some selection
and enrichment process. For example, by repeated
cycles of affinity chromatography and PCR
amplification of the molecules bound to the ligand
on the column, Ellington and Szostak (1990)
25 estimated that 1 in 10^{10} RNA molecules folded in
such a way as to bind a given ligand. DNA
molecules with such ligand-binding behavior have
been isolated (Ellington and Szostak, 1992; Bock et
al, 1992).

30   *Computer assisted drug design*
Computer modeling technology allows
visualization of the three-dimensional atomic
structure of a selected molecule and the rational
design of new compounds that will interact with the
35 molecule. The three-dimensional construct
typically depends on data from x-ray
crystallographic analyses or NMR imaging of the
selected molecule. The molecular dynamics require

force field data. The computer graphics systems
enable prediction of how a new compound will link
to the target molecule and allow experimental
manipulation of the structures of the compound and
5   target molecule to perfect binding specificity.
Prediction of what the molecule-compound
interaction will be when small changes are made in
one or both requires molecular mechanics software
and computationally intensive computers, usually
10   coupled with user-friendly, menu-driven interfaces
between the molecular design program and the user.
Examples of molecular modelling systems
are the CHARMm and QUANTA programs, Polygen
Corporation, Waltham, MA. CHARMm performs the
15   energy minimization and molecular dynamics
functions. QUANTA performs the construction,
graphic modelling and analysis of molecular
structure. QUANTA allows interactive construction,
modification, visualization, and analysis of the
20   behavior of molecules with each other.
A number of articles review computer
modeling of drugs interactive with specific
proteins, such as Rotivinen, et al., (1988) <u>Acta
Pharmaceutica Fennica</u> 97, 159-166; Ripka, <u>New
25   Scientist</u> 54-57 (June 16, 1988); McKinaly and
Rossmann, (1989) <u>Annu. Rev. Pharmacol. Toxiciol.</u>
29, 111-122; Perry and Davies, <u>QSAR: Quantitative
Structure-Activity Relationships in Drug Design</u> pp.
189-193 (Alan R. Liss, Inc. 1989); Lewis and Dean,
30   (1989) <u>Proc. R. Soc. Lond.</u> 236, 125-140 and 141-
162; and, with respect to a model receptor for
nucleic acid components, Askew, et al., (1989) <u>J.
Am. Chem. Soc.</u> 111, 1082-1090. Other computer
programs that screen and graphically depict
35   chemicals are available from companies such as
BioDesign, Inc., Pasadena, CA., Allelix, Inc,
Mississauga, Ontario, Canada, and Hypercube, Inc.,

27

Cambridge, Ontario. Although these are primarily
designed for application to drugs specific to
particular proteins, they can be adapted to design
of drugs specific to regions of DNA or RNA, once
5   that region is identified.

Although described above with reference
to design and generation of compounds which could
alter binding, one could also screen libraries of
known compounds, including natural products or
10   synthetic chemicals, and biologically active
materials, including proteins, for compounds which
are inhibitors or activators.

Generation of nucleic acid regulators

Nucleic acid molecules containing the 5'
15   regulatory sequences of the receptor genes can be
used to regulate or inhibit gene expression *in*
vivo. Vectors, including both plasmid and
eukaryotic viral vectors, may be used to express a
particular recombinant 5' flanking region-gene
20   construct in cells depending on the preference and
judgment of the skilled practitioner (see, e.g.,
Sambrook et al., Chapter 16). Furthermore, a
number of viral and nonviral vectors are being
developed that enable the introduction of nucleic
25   acid sequences *in vivo* (see, e.g., Mulligan, (1993)
Science, 260, 926-932; United States Patent No.
4,980,286; United States Patent No. 4,868,116;
incorporated herein by reference). Recently, a
delivery system was developed in which nucleic acid
30   is encapsulated in cationic liposomes which can be
injected intravenously into a mammal. This system
has been used to introduce DNA into the cells of
multiple tissues of adult mice, including
endothelium and bone marrow (see, e.g., Zhu et al.,
35   (1993) Science 261, 209-211; incorporated herein by
reference).

The 5'-flanking sequences of the receptor
gene can also be used to inhibit the expression of
the receptor. For example, an antisense RNA of all
or a portion of the 5' flanking region of the
5   receptor gene can be used to inhibit expression of
the receptor in vivo. Expression vectors (e.g.,
retroviral expression vectors) are already
available in the art which can be used to generate
an antisense RNA of a selected DNA sequence which
10   is expressed in a cell (see, e.g., U.S. Patent No.
4,868,116; U.S. Patent No. 4,980,286).
Accordingly, DNA containing all or a portion of the
sequence of the 5' flanking region of the receptor
gene can be inserted into an appropriate expression
15   vector so that upon passage into the cell, the
transcription of the inserted DNA yields an
antisense RNA that is complementary to the mRNA
transcript of the receptor protein gene normally
found in the cell. This antisense RNA transcript
20   of the inserted DNA can then base-pair with the
normal mRNA transcript found in the cell and
thereby prevent the mRNA from being translated. It
is of course necessary to select sequences of the
5' flanking region that are downstream from the
25   transcriptional start sites for the receptor
protein gene to ensure that the antisense RNA
contains complementary sequences present on the
mRNA.

Antisense RNA can be generated in vitro
30   also, and then inserted into cells.
Oligonucleotides can be synthesized on an automated
synthesizer (e.g., Model 8700 automated synthesizer
of Milligen-Biosearch, Burlington, MA or ABI Model
380B). In addition, antisense
35   deoxyoligonucleotides have been shown to be
effective in inhibiting gene transcription and
viral replication (see e.g., Zamecnik et al.,

(1978) Proc. Natl. Acad. Sci. USA 75, 280-284;
Zamecnik et al., (1986) Proc. Natl. Acad. Sci., 83,
4143-4146; Wickstrom et al., (1988) Proc. Natl.
Acad. Sci. USA 85, 1028-1032; Crooke, (1993) FASEB

5   J. 7, 533-539. Furthermore, recent work has shown
that improved inhibition of expression of a gene by
antisense oligonucleotides is possible if the
antisense oligonucleotides contain modified
nucleotides (see, e.g., Offensperger et. al.,

10   (1993) EMBO J. 12, 1257-1262 (in vivo inhibition of
duck hepatitis B viral replication and gene
expression by antisense phosphorothioate
oligodeoxynucleotides); PCT WO 93/01286 Rosenberg
et al., (synthesis of sulfurthioate

15   oligonucleotides); Agrawal et al., (1988) Proc.
Natl. Acad. Sci USA 85, 7079-7083 (synthesis of
antisense oligonucleoside phosphoramidates and
phosphorothioates to inhibit replication of human
immunodeficiency virus-1); Sarin et al., (1989)

20   Proc. Natl. Acad. Sci USA 85, 7448-7794 (synthesis
of antisense methylphosphonate oligonucleotides);
Shaw et al., (1991) Nucleic Acids Res. 19, 747-750
(synthesis of 3' exonuclease-resistant
oligonucleotides containing 3' terminal

25   phosphoroamidate modifications); incorporated
herein by reference).
          The sequences of the 5' flanking region
of receptor protein gene can also be used in triple
helix (triplex) gene therapy. Oligonucleotides

30   complementary to gene promoter sequences on one of
the strands of the DNA have been shown to bind
promoter and regulatory sequences to form local
triple nucleic acid helices which block
transcription of the gene (see, e.g., Maher et al.,

35   (1989) Science 245, 725-730; Orson et al., (1991)
Nucl. Acids Res. 19, 3435-3441; Postal et al.,
(1991) Proc. Natl. Acad. Sci. USA 88, 8227-8231;

Cooney et al., (1988) Science 241, 456-459; Young
et al., (1991) Proc. Natl. Acad. Sci. USA 88,
10023-10026; Duval-Valentin et al., (1992) Proc.
Natl. Acad. Sci. USA 89, 504-508; Blume et al.,
5   (1992) Nucl. Acids Res. 20, 1777-1784; Grigoriev et
al., (1992) J. Biol. Chem. 267, 3389-3395.

Recently, both theoretical calculations
and empirical findings have been reported which
provide guidance for the design of oligonucleotides
10   for use in oligonucleotide-directed triple helix
formation to inhibit gene expression. For example,
oligonucleotides should generally be greater than
14 nucleotides in length to ensure target sequence
specificity (see, e.g., Maher et al., (1989);
15   Grigoriev et al., (1992)). Also, many cells avidly
take up oligonucleotides that are less than 50
nucleotides in length (see, e.g., Orson et al.,
(1991); Holt et al., (1988) Mol. Cell. Biol. 8,
963-973; Wickstrom et al., (1988) Proc. Natl. Acad.
20   Sci. USA 85, 1028-1032). To reduce susceptibility
to intracellular degradation, for example by 3'
exonucleases, a free amine can be introduced to a
3' terminal hydroxyl group of oligonucleotides
without loss of sequence binding specificity (Orson
25   et al., 1991). Furthermore, more stable triplexes
are formed if any cytosines that may be present in
the oligonucleotide are methylated, and also if an
intercalating agent, such as an acridine
derivative, is covalently attached to a 5' terminal
30   phosphate (e.g., via a pentamethylene bridge);
again without loss of sequence specificity (Maher
et al., (1989); Grigoriev et al., (1992).

Methods to produce or synthesize
oligonucleotides are well known in the art. Such
35   methods can range from standard enzymatic digestion
followed by nucleotide fragment isolation (see
e.g., Sambrook et al., Chapters 5, 6) to purely

synthetic methods, for example, by the cyanoethyl
phosphoramidite method using a Milligen or Beckman
System 1Plus DNA synthesizer (see also, Ikuta et
al., (1984) Ann. Rev. Biochem. 53, 323-356
5   (phosphotriester and phosphite-triester methods);
Narang et al., (1980) Methods Enzymol., 65, 610-620
(phosphotriester method). Accordingly, DNA
sequences of the 5' flanking region of the receptor
protein gene described herein can be used to design
10   and construct oligonucleotides including a DNA
sequence consisting essentially of at least 15
consecutive nucleotides, with or without base
modifications or intercalating agent derivatives,
for use in forming triple helices specifically
15   within the 5' flanking region of a receptor protein
gene in order to inhibit expression of the gene.
       In some cases it may be advantageous to
insert enhancers or multiple copies of the
regulatory sequences into an expression system to
20   facilitate screening of methods and reagents for
manipulation of expression.
       Preparation of Receptor Protein Fragments
       Compounds which are effective for
blocking binding of the receptor can also consist
25   of fragments of the receptor proteins, expressed
recombinantly and cleaved by enzymatic digest or
expressed from a sequence encoding a peptide of
less than the full-length receptor protein. These
will typically be soluble proteins, i.e., not
30   including the transmembrane and cytoplasmic
regions, although smaller portions determined in
the assays described above to inhibit or compete
for binding to the receptor proteins can also be
utilized. It is a routine matter to make
35   appropriate receptor protein fragments, test for
binding, and then utilize. The preferred fragments
are of human origin, in order to minimize potential

immunological response.   The peptides can be as
short as five to eight amino acids in length and
are easily prepared by standard techniques.   They
can also be modified to increase in vivo half-life,
by chemical modification of the amino acids or by
attachment to a carrier molecule or inert
substrate.   Based on studies with other peptide
fragments blocking receptor binding, the IC_{50}, the
dose of peptide required to inhibit binding by 50%,
ranges from about 1 μM to greater than 10 mM,
depending on the peptide size and folding.   These
ranges are well within the effective concentrations
for the in vivo administration of peptides, based
on comparison with the RGD-containing peptides,
described, for example, in U.S. Patent No.
4,792,525 to Ruoslaghti, et al., used in vivo to
alter cell attachment and phagocytosis. The
peptides can also be conjugated to a carrier
protein such as keyhole limpet hemocyanin by its N-
terminal cysteine by standard procedures such as
the commercial Imject kit from Pierce Chemicals or
expressed as a fusion protein, which may have
increased efficacy.

        As noted above, the peptides can be
prepared by proteolytic cleavage of the receptor
proteins, or, preferably, by synthetic means.
These methods are known to those skilled in the
art.   An example is the solid phase synthesis
described by J. Merrifield, (1964) <u>J. Am. Chem.
Soc.</u> 85, 2149, used in U.S. Patent No. 4,792,525,
and described in U.S. Patent No. 4,244,946, wherein
a protected alpha-amino acid is coupled to a
suitable resin, to initiate synthesis of a peptide
starting from the C-terminus of the peptide. Other
methods of synthesis are described in U.S. Patent
No. 4,305,872 and 4,316,891. These methods can be
used to synthesize peptides having identical

sequence to the receptor proteins described herein,
or substitutions or additions of amino acids, which
can be screened for activity as described above.

The peptide can also be administered as a
5   pharmaceutically acceptable acid- or base- addition
salt, formed by reaction with inorganic acids such
as hydrochloric acid, hydrobromic acid, perchloric
acid, nitric acid, thiocyanic acid, sulfuric acid,
and phosphoric acid, and organic acids such as
10   formic acid, acetic acid, propionic acid, glycolic
acid, lactic acid, pyruvic acid, oxalic acid,
malonic acid, succinic acid, maleic acid, and
fumaric acid, or by reaction with an inorganic base
such as sodium hydroxide, ammonium hydroxide,
15   potassium hydroxide, and organic bases such as
mono-, di-, trialkyl and aryl amines and
substituted ethanolamines.

Peptides containing cyclopropyl amino
acids, or amino acids derivatized in a similar
20   fashion, can also be used. These peptides retain
their original activity but have increased half-
lives *in vivo*. Methods known for modifying amino
acids, and their use, are known to those skilled in
the art, for example, as described in U.S. Patent
25   No. 4,629,784 to Stammer.

The peptides are generally active when
administered parenterally in amounts above about 1
μg/kg of body weight. Based on extrapolation from
other proteins, for treatment of most inflammatory
30   disorders, the dosage range will be between 0.1 to
70 mg/kg of body weight. This dosage will be
dependent, in part, on whether one or more peptides
are administered.

Pharmaceutical Compositions

35   Compounds which alter receptor protein
binding are preferably administered in a
pharmaceutically acceptable vehicle. Suitable

pharmaceutical vehicles are known to those skilled
in the art. For parenteral administration, the
compound will usually be dissolved or suspended in
sterile water or saline. For enteral
5   administration, the compound will be incorporated
into an inert carrier in tablet, liquid, or
capsular form. Suitable carriers may be starches
or sugars and include lubricants, flavorings,
binders, and other materials of the same nature.
10   The compounds can also be administered locally by
topical application of a solution, cream, gel, or
polymeric material (for example, a Pluronic™,
BASF).

        Alternatively, the compound may be
15   administered in liposomes or microspheres (or
microparticles). Methods for preparing liposomes
and microspheres for administration to a patient
are known to those skilled in the art. U.S. Patent
No. 4,789,734 describe methods for encapsulating
20   biological materials in liposomes. Essentially,
the material is dissolved in an aqueous solution,
the appropriate phospholipids and lipids added,
along with surfactants if required, and the
material dialyzed or sonicated, as necessary. A
25   review of known methods is by G. Gregoriadis,
Chapter 14. "Liposomes", Drug Carriers in Biology
and Medicine pp. 287-341 (Academic Press, 1979).
Microspheres formed of polymers or proteins are
well known to those skilled in the art, and can be
30   tailored for passage through the gastrointestinal
tract directly into the bloodstream.
Alternatively, the compound can be incorporated and
the microspheres, or composite of microspheres,
implanted for slow release over a period of time,
35   ranging from days to months. See, for example,
U.S. Patent Nos. 4,906,474, 4,925,673, and
3,625,214.

Disorders to be treated

As described herein, a variety of compounds can be used to inhibit or enhance expression of the EPCR. The nature of the disorder will determine if the expression should be enhanced or inhibited. For example, based on the studies involving the use of an anti-protein C antibody in combination with cytokine, it should be possible to treat solid tumors by enhancing an inflammatory response involving blocking of protein C or activated protein C binding to an endothelial cell protein C/activated protein C receptor by administering to a patient in need of treatment thereof an amount of a compound blocking binding of protein C or activated protein C to the receptor. Similarly, it should be possible to treat disorders such as gram negative sepsis, stroke, thrombosis, septic shock, adult respiratory distress syndrome, and pulmonary emboli using a method for inhibiting an inflammatory response involving administration of EPCR or EPCR fragments or substances that upregulate EPCR expression to a patient in need of treatment thereof.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT: Oklahoma Medical Research Foundation

    (ii) TITLE OF INVENTION: Cloning and Regulation of an Endothelial Cell Protein C/Activated Protein C Receptor

    (iii) NUMBER OF SEQUENCES: 2

    (iv) CORRESPONDENCE ADDRESS:

        (A) ADDRESSEE: Patrea L. Pabst

        (B) STREET: 2800 One Atlantic Center

            1201 West Peachtree Street

        (C) CITY: Atlanta

        (D) STATE: Georgia

        (E) COUNTRY: USA

        (F) ZIP: 30309-3450

    (v) COMPUTER READABLE FORM:

        (A) MEDIUM TYPE: Floppy disk

        (B) COMPUTER: IBM PC compatible

        (C) OPERATING SYSTEM: PC-DOS/MS-DOS

        (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

  (viii) ATTORNEY/AGENT INFORMATION:

        (A) NAME: Pabst, Patrea L.

        (B) REGISTRATION NUMBER: 31,284

        (C) REFERENCE/DOCKET NUMBER: OMRF152

    (ix) TELECOMMUNICATION INFORMATION:

        (A) TELEPHONE: (404) 873-8794

        (B) TELEFAX: (404) 873-8795

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 1302 base pairs

        (B) TYPE: nucleic acid

        (C) STRANDEDNESS: single

        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (iii) HYPOTHETICAL: NO

    (iv) ANTI-SENSE: NO

    (ix) FEATURE:

        (A) NAME/KEY: misc_feature

        (B) LOCATION: 1..1302
        (D) OTHER INFORMATION: /note= "Nucleotides 25 through 738 encode the
Endothelial Cell Protein Receptor of Sequence ID No. 2."
    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CAGGTCCGGA GCCTCAACTT CAGGATGTTG ACAACATTGC TGCCGATACT GCTGCTGTCT   60

GGCTGGGCCT TTTGTAGCCA AGAGGCCTCA GATGGCCTCC AAAGACTTCA TATGCTCCAG   120

ATCTCCTACT TCCGCGACCC CTATCACGTG TGGTACCAGG GCAACGCGTC GCTGGGGGGA   180

CACCTAACGC ACGTGCTGGA AGGCCCAGAC ACCAACACCA CGATCATTCA GCTGCAGCCC   240

TTGCAGGAGC CCGAGAGCTG GGCGCGGCACG CAGAGTGGCC TGCAGTCCTA CCTGCTCCAG   300

TTCCACGGCC TCGTGCGCCT GGTGCACCAG GAGCGGACCT TGGCCTTTCC TCTGACCATC   360

CGCTGCTTCC TGGGCTGTGA GCTGCCTCCC GAGGGCTCTA GAGCCCATGT CTTCTTCGAA   420

GTGGCTGTGA ATGGGAGCTC CTTTGTGAGT TTCCGGCCGG AGAGAGCCTT GTGGCAGGCA   480

GACACCCAGG TCACCTCCGG AGTGGTCACC TTCACCCTGC AGCAGCTCAA TGCCTACAAC   540

CGCACTCGGT ATGAACTGCG GGAATTCCTG GAGGACACCT GTGTGCAGTA TGTGCAGAAA   600

CATATTTCCG CGGAAAACAC GAAAGGGAGC CAAACAAGCC GCTCCTACAC TTCGCTGGTC   660

CTGGGCGTCC TGGTGGGCGG TTTCATCATT GCTGGTGTGG CTGTAGGCAT CTTCCTGTGC   720

ACAGGTGGAC GGCGATGTTA ATTACTCTCC AGCCCCGTCA GAAGGGGCTG GATTGATGGA   780

GGCTGGCAAG GGAAAGTTTC AGCTCACTGT GAAGCCAGAC TCCCAACTG AAACACCAGA   840

AGGTTTGGAG TGACAGCTCC TTTCTTCTCC CACATCTGCC CACTGAAGAT TTGAGGGAGG   900

GGAGATGGAG AGGAGAGGTG GACAAAGTAC TTGGTTTGCT AAGAACCTAA GAACGTGTAT   960

```
GCTTTGCTGA ATTAGTCTGA TAAGTGAATG TTTATCTATC TTTGTGGAAA ACAGATAATG    1020

GAGTTGGGGC AGGAAGCCTA TGCGCCATCC TCCAAAGACA GACAGAATCA CCTGAGGCGT    1080

TCAAAAGATA TAACCAAATA AACAAGTCAT CCACAATCAA AATACAACAT TCAATACTTC    1140

CAGGTGTGTC AGACTTGGGA TGGGACGCTG ATATAATAGG GTAGAAAGAA GTAACACGAA    1200

GAAGTGGTGG AAATGTAAAA TCCAAGTAAA TCCAAGTCAT ATGGCAGTGA TCAATTATTA ATCAATTAAT    1260

AATATTAATA AATTTCTTAT ATTTAAAAA AAAAAAAAA AA    1302
```

(2) INFORMATION FOR SEQ ID NO:2:
    (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 238 amino acids
      (B) TYPE: amino acid
      (D) TOPOLOGY: linear
    (ii) MOLECULE TYPE: protein
   (iii) HYPOTHETICAL: NO
    (ix) FEATURE:
      (A) NAME/KEY: misc_feature
      (B) LOCATION: 1..365
      (D) OTHER INFORMATION: /note= "Endothelial Cell Protein Receptor encoded by nucleotides 1 through 1302 of Sequence ID No. 1."
    (ix) FEATURE:
      (A) NAME/KEY: Modified-site
      (B) LOCATION: 1..15
      (D) OTHER INFORMATION: /note= "Amino acids 1-15 represent a putative signal sequence."
    (ix) FEATURE:
      (A) NAME/KEY: Domain
      (B) LOCATION: 211..236
      (D) OTHER INFORMATION: /note= "Amino acids 211-236 represent a putative transmembrane domain."
    (ix) FEATURE:
      (A) NAME/KEY: Activ -site
      (B) LOCATION: 47..174

39

(D) OTHER INFORMATION: /note= "Amino acids 47-49, 64-66,
    136-138 and 172-174 represent potential
    N-glycosylation sites."

(ix) FEATURE:
    (A) NAME/KEY: Modified-site
    (B) LOCATION: 17..186
    (D) OTHER INFORMATION: /note= "Amino acids 17, 114, 118
        and 186 represent extracellular cysteine
        residues."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Leu Thr Thr Leu Leu Pro Ile Leu Leu Leu Ser Gly Trp Ala Phe
1               5                   10                  15

Cys Ser Gln Asp Ala Ser Asp Gly Leu Gln Arg Leu His Met Leu Gln
        20                  25                  30

Ile Ser Tyr Phe Arg Asp Pro Tyr His Val Trp Tyr Gln Gly Asn Ala
        35                  40                  45

Ser Leu Gly Gly His Leu Thr His Val Leu Glu Gly Pro Asp Thr Asn
    50                  55                  60

Thr Thr Ile Ile Gln Leu Gln Pro Leu Gln Glu Pro Glu Ser Trp Ala
65                  70                  75                  80

Arg Thr Gln Ser Gly Leu Gln Ser Tyr Leu Leu Gln Phe His Gly Leu
        85                  90                  95

Val Arg Leu Val His Gln Glu Arg Thr Leu Ala Phe Pro Leu Thr Ile
        100                 105                 110

Arg Cys Phe Leu Gly Cys Glu Leu Pro Pro Glu Gly Ser Arg Ala His
        115                 120                 125

Val Phe Phe Glu Val Ala Val Asn Gly Ser Ser Val Ser Phe Arg
        130                 135                 140
```

Pro Glu Arg Ala Leu Trp Gln Ala Asp Thr Gln Val Thr Ser Gly Val
145   150   155   160

Val Thr Phe Thr Leu Gln Gln Leu Asn Ala Tyr Asn Arg Thr Arg Tyr
                    165   170   175

Glu Leu Arg Glu Phe Leu Glu Asp Thr Cys Val Gln Tyr Val Gln Lys
180   185   190

His Ile Ser Ala Glu Asn Thr Lys Gly Ser Gln Thr Ser Arg Ser Tyr
                    195   200   205

Thr Ser Leu Val Leu Gly Val Leu Val Gly Gly Phe Ile Ile Ala Gly
210   215   220

Val Ala Val Gly Ile Phe Leu Cys Thr Gly Gly Arg Arg Cys
225   230   235

41

We claim.

1.   An isolated endothelial cell protein C/activated protein C receptor.

2.   The receptor of claim 1 encoded by the nucleotide sequence of Sequence ID No. 1 and degenerative sequences thereof and sequence having conservative substitutions, additions or deletions thereof hybridizing to Sequence ID No. 1 under stringent conditions, which encode the receptor.

3.   The receptor of claim 1 having the amino acid sequence of Sequence ID No. 2 or a sequence having conservative substitutions, additions or deletions thereof.

4.   The receptor of claim 1 expressed on the surface of a non-human cell or a non-endothelial cell.

5.   The receptor of claim 1 in soluble form.

6.   The receptor of claim 5 lacking at least a portion of the transmembrane region.

7.   A nucleotide sequence encoding an endothelial cell protein C/activated protein C receptor.

8.   The sequence of claim 7 having the nucleotide sequence of Sequence ID No. 1 or degenerative sequences thereof and sequence having conservative substitutions, additions or deletions thereof and hybridizing under stringent conditions to Sequence ID No. 1.

9.   The sequence of claim 7 encoding the amino acid sequence of Sequence ID No. 2 or a sequence having conservative substitutions, additions or deletions thereof.

10.   The sequence of claim 7 further comprising an expression vector.

11.   The sequence of claim 10 further comprising an expression host.

12. The sequence of claim 10 expressed
on the surface of a non-human cell or a non-
endothelial cell.

13. The sequence of claim 7 encoding a
soluble form of the receptor.

14. The sequence of claim 7 encoding a
fragment of the receptor of at least fourteen
consecutive nucleotides in length.

15. The sequence of claim 14 labelled
with a detectable label.

16. A method for enhancing an
inflammatory response involving blocking of protein
C or activated protein C binding to an endothelial
cell protein C/activated protein C receptor
comprising administering to a patient in need of
treatment thereof an amount of a compound blocking
binding of protein C or activated protein C to the
receptor. tumors

17. The method of claim 16 wherein the
compound is selected from the group consisting of
antibodies and fragments of antibodies to the
receptor, nucleic acid sequences inhibiting
expression of the receptor, and synthetic or
natural compounds other than proteins, peptides or
nucleic acid sequences which inhibit binding.

18. A method for inhibiting an
inflammatory response involving administration of a
compound selected from the group consisting of EPCR
or EPCR fragments and substances that upregulate
EPCR expression to a patient in need of treatment
thereof.

19. An antibody or antibody fragment
specifically immunoreactive with a unique epitope
of an isolated endothelial cell protein C/activated
protein C receptor.

20. The antibody of claim 19 wherein the
receptor is encoded by the nucleotide sequence of

Sequence ID No. 1 and degenerative sequences
thereof and sequence having conservative
substitutions, additions or deletions thereof and
hybridizing to Sequence ID No. 1 under stringent
conditions.

21. The antibody of claim 19 wherein the
receptor has the amino acid sequence of Sequence ID
No. 2 or a sequence having conservative
substitutions, additions or deletions thereof.

22. A method for screening for a
compound which alters the binding of an endothelial
receptor protein to protein C or activated protein
C comprising providing an assay for binding of
protein C or activated protein C to the receptor
protein,

adding the compound to be tested to the
assay, and

determining if the amount of protein C or
activated protein C which is bound to the receptor
protein is altered as compared to binding in the
absence of the compound to be tested.

23. A method for screening patients for
abnormal receptor protein activity or function
comprising

determining the presence of an
endothelial cell surface receptor binding protein C
and activated protein C, and

comparing the receptor to determine if
the quantity present or the function of the
receptor is equivalent to that present in normal
cells.

FIG. 1a

FIG. 1b

FIG. 1c

FIG. 2a

FIG. 2b

FIG. 2c

FIG. 2d

FIG. 3a

FIG. 3b

FIG. 4a

CAGGTCCGGAGCCTCAACTTCAGGATGTTGACAACATTGCTGCCGATACTGCTGCTGTCT 60
                              M L T T L P I L L L S   12

GGCTGGGCCTTTGTAGCCAAGAGACGCCTCAGATGGCCTCCAAAGACTTCATATGCTCCAG 120
G W A F C S Q D A S D G L Q R L H M L Q 32

ATCTCCTACTTCCGGACCCCTATCACGTGTGGTACCAGGGCAACGCG TCGCTGGGGGGA 180
I S Y F R D P Y H V W Y Q G N A S L G G 52

CACCTAACGCACGTGCTGGAAGGCCCAGACAACACCAACACCACGATCATTCAGCTGCAGCCC 240
H L T H V L E G P D T N T T I I Q L Q P 72

TTGCAGGAGCCCGAGAGCTGGGCGCGCACGCAGAGTGGCCTGCAGTCCTACCTGCTCCAG 300
L Q E P E S W A R T Q S G L Q S Y L L Q 92

TTCCACGGCCTCGTGCGCCTGGTGCACCAGGAGCGGACCTTGGCCTTTCCTCTGACCATC 360
F H G L V R L V H Q E R T L A F P L T I 112

CGCTGCTTCCTGGGCTGTGAGCTGCCTCCCGAGGGCTCTAGAGCCCATGTCTTCTTCGAA 420
R C F L G E L P P E G S R A H V F F E 132

SUBSTITUTE SHEET (RULE 26)

FIG. 4b

GTGGCTGTGAATGGGAGCTCCTTGTGAGTTCCGGCCGGAGAGAGCCTTGTGGCAGGCA   480
 V   A   V   N   G   S   S   F   V   S   F   R   P   E   R   A   L   W   Q   A   152

GACACCCAGGTCACCTCCGGAGTGGTCACCTTCACCCTGCAGCAGTCAATGCCTACAAC   540
 D   T   Q   V   T   S   G   V   V   T   F   T   L   Q   Q   L   N   A   Y   N   172

CGGCACTCGGTATGAACTGCGGGAATTCCTGGAGGACACTGTGTGCAGTATGTGCAGAAA   600
 R   T   R   Y   E   L   R   E   F   L   E   D   T   C   V   Q   Y   V   Q   K   192

CATATTCCGCGGAAAAACACGAAAGGGAGCCAAACAAGCCGTCCTACACTTCGCTGGTC   660
 H   I   S   A   E   N   T   K   G   S   Q   T   S   R   S   Y   T   S   L   V   212

CTGGGCGTCCTGGTGGGGGTTCATCATTGCTGGTGGTGTGGCTGTAGGCATCTTCCTGTGC   720
 L   G   V   L   V   G   G   F   I   I   A   G   V   A   V   G   I   F   L   C   232

ACAGGTGGACGGCGGATGTTAATTACTCTCCAGCCCCGTCAGAAAGGGGCTGGATTGATGGA   780
 T   G   G   R   R   C   *   238

FIG. 4c

GGCTGGCAAGGGAAAGTTTCAGCTCACTGTGAAGCCAGACTCCCAACTGAAACACCAGA 840

AGGTTTGGAGTGACAGCTCCTTCTTCTCCCACATCTGCCCACTGAAGATTTGAGGGAGG 900

GGAGATGGAGGAGGAGAGGTGGACAAAGTACTTGGTTGCTAAGAACCTAAGAACGTGTAT 960

GCTTTGCTGAATTAGTCTGATAAGTGAATGTTTATCTTTGTGGAAAACAGATAATG 1020

GAGTTGGGGCAGGAAGCCTATGCGCCATCCTCCAAAGACACAGAGAATCACCTGAGGCGT 1080

TCAAAAGATATAACCAAATAAACAAGTCATCCACAATCAAAATACAACATTCAATACTTC 1140

CAGGTGTGTCAGACTTGGGATGGGACGCTGATATAATAGGGTAGAAAGAAGTAACACGAA 1200

GAAGTGGTGGAAATGTAAAATCCAAGTCATATGGCCAGTGATCAATTATTAATCAATTAAT 1260

AATATTAATAAATTCTTATATTTAAAAAAAAAAAAAAAA 1302

SIGNAL SEQUENCE

TRANSMEMBRANE

HYDRO-PHOBIC

HYDRO-PHILIC

FIG. 5a

EPCR   MLTT..LLPILLLSGWAFCSQDASDGLQRLHMLQISYFRDLPYHVWYQGNASLGGHLTHVL
CCD41 MLTKFLLLLLLLPGCAFVTPMAPKAAYAPDLL...FPRLPPSCEASGQRSLGKLLTHTL
CD1d   MGC...LLFLLLWALLQAWGSAEVPQRLFPLRCLQISSFANSSWTRTDGLAWLGELQTHSW
MCD1.2 MRY..LPCLLLWAFLQVWGQSEVQQKNYTFRCLQTSSFANISWSRTDSLILLGDLQTHRW

EPCR   EGPDTNTTIIQLQPLQEPESWARTQSGLQSYLLQFHGLVRLVHQER.....TLAFPLTI
CCD41 EGPSQNVTILQLQPWQDPESWERTESGLQIMLTHQFESLVKLVYRER...KENVFFPLTV
CD1d   SN..DSDTVRSLRPWSQGTFSDQQWETLQHIFRVYRSSFTRDVKEFAKML..RLSYPLEL
MCD1.2 SN..DSATISFTKPWSQGKLSNQQWEKLQHMFQVYRVSFTRDIQELVKMMSPKEDYPLEL

EPCR   RCLFLGCEL.P...PEGSRAHVFFEVAVNGSSFVSFRPERALWQADTQVTSGVVTFTLQQL
CCD41 SCSLGCEL.PEEEEGSEPHVFFDVAVNGSAFVSFRPKTAVWVSGSQEPSKAANFTLKQL
CD1d   QVSAGCEVHP....GNASNNFFHVAFQGKDILSFQGTS...WEP.TQEAPLWLNLAIQVL
MCD1.2 QLSTGCEMYP.....GNASESEFHVAFQGKYAVURFRGTS..WQR.VLGAPSWLDLPIKVL

FIG. 5b

```
                    *     *  *  ***  **   *   *           *  *   **   *
EPCR    * *  *   *  *  ***  ***  NAYNRTRYELREFLEDTCVQYV..........QKHISAENTKGSQTS..................
CCD41                            NAYNRTRYELQEFLQDTCVEFL.........ENHITTQNMKGSQTG..................
CD1d                             NQDKWTRETVQWLLNGTCPQFVSGLLESGKSELKKQVKPKAWLSRGPSPGPGRLLLVCHVSG
MCD1.2                           NADQGTSATVQTLLNDTWPQFARGLLEAGKSDLEKQEKPVAWLSSVPSSAHGHLQLVCHVSG

EPCR    ........................................................................
CCD41   ........................................................................
CD1d    FYPKPVWVKWMRGEQEQQGTQPGDILPNADETWYLRATLDVVAGEAAGLSCRVKHSSLEG
MCD1.2  FYPKPVWVMWMRGDQEQQGTHRGDFLPNADETWYLQATLDVVEAGEEAGLACRVKHSSLGG

EPCR    ****   ***           *                            **
CCD41   ........RSYTSLVLGVLVGGFIIAGVAVGIFLCTGGRRC
CD1d    .......RSYTSLVLGILMGCFIIAGVAVGIFMCTSGRGLLII
CD1d    QDIVLYWGGSYTSMGLIALAVLACLLFLLIVGFTS.RFKIRQTSYQGVL
MCD1.2  QDIILYWDARQAPVGLIVFIVLIMLVVVGAVVYYI.WRRRSAYQDIR
```

EPCR
CCD41
CD1d
MCD1.2

1 MLTTLLP..ILLLSGWAFCSQDASDGLQRLHMLQISYFRDPYHVWYQGNA48
  ||⁚|| ⁚|||⁚|⁚| |||⁚|⁚|| ||||||⁚|⁚⁚||⁚⁚||||
1 MLTKFLPLLLLLLPGCALCN...SDGSQSLHMLQISYFQDHHHVRHQGNA 47

49 SLGGHLTHVLEGPDTNTTIIQLQPLQEPESWARTQSGLQSYLLQFHGLVR 98
   ||| |||⁚|||⁚|||⁚||||⁚|⁚|||⁚||⁚||||⁚|||||⁚⁚||⁚
48 SLGKLLTHTLEGPSQNVTILQLQPWQDPESWERTESGLQIYLTQFESLVK 97

99 LVHQER..TLAFPLTIRCFLGCELP...PEGSRAHVFFEVAVNGSSFVSF 143
   ||⁚|| ⁚⁚||||⁚| ||||| ⁚|||⁚|||⁚||||||⁚|||
98 LVYRERKENVFFPLTVSCSLGCELPEEEEEGSEPHVFFDVAVNGSAFVSF 147

144RPERALWQADTQVTSGVVTFTLQQLNAYNRTRYELREFLEDTCVQYVQKH193
   ||⁚|⁚|⁚⁚⁚|⁚|⁚⁚||⁚|||||||||||⁚|||⁚|||⁚⁚⁚⁚⁚|
148 RPKTAVWVSGSQEPSKAANFTLKQLNAYNRTRYELQEFLQDTCVEFLENH 197

194 ISAENTKGSQTSRSYTSLVLGVLVGGFIIAGVAVGIFLCTGGRRC 238
    |⁚⁚||||||⁚|||||||||⁚|⁚|⁚|||||||||||⁚||⁚||||
198 ITTQNMKGSQTGRSYTSLVLGILMGCFIIAGVAVGIFMCTSGRRC 242

FIG. 6

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C12N15/12   C07K14/705   A61K39/395   C12N15/11   A61K38/17
C07K16/28   G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   C12N   C07K   A61K   G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category°	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 42, 21 October 1994 BALTIMORE, MD, USA, pages 26486-26491, K. FUKUDOME ET AL. 'Identification, cloning, and regulation of a novel endothelial cell protein C/activated protein C receptor.' see the whole document --- -/--	1-3,7-11

[X] Further documents are listed in the continuation of box C.   [ ] Patent family members are listed in annex.

Date of the actual completion of the international search	Date of mailing of the international search report
2 January 1996	26.01.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Nooij, F

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	CIRCULATION, vol. 90, no. 4 part 2, October 1994 NEW YORK, NY, USA, page I-N K. FUKUDOME ET AL. 'Identification, cloning, and regulation of a novel endothelial cell protein C/activated protein C receptor.' see the whole document ---	1-3,7-11
P,X	CIRCULATION, vol. 90, no. 4 part 2, October 1994 NEW YORK, NY, USA, page I133 K. FUKUDOME ET AL. 'Identification, cloning and regulation of a novel endothelial cell protein C/activated protein C receptor.' see abstract 0707 ---	1-3,7-11
P,X	THROMBOSIS AND HAEMOSTASIS, vol. 72, no. 3, September 1994 STUTTGART, GERMANY, pages 465-474, N. BANGALORE ET AL. 'High affinity binding sites for activated protein C and protein C on cultured human umbilical vein endothelial cells.' see summary see page 472, right column, line 9 - line 50 ---	1-3,7-11
P,X	THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 10, 10 March 1995 BALTIMORE, MD, USA, pages 5571-5577, K. FUKUDOME ET AL. 'Molecular cloning and expression of murine and bovine endothelial cell protein C/activated protein C receptor (EPCR).' see abstract see figures 1-5 -----	1-4,7-12

1

INTERNATIONAL SEARCH REPORT

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 16-18
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although these claims are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)